

ADPRT INHIBITORS AND HYPERTHERMIA AS RADIOSENSITIZERS

**Mechanistic studies using human leukocytes in vitro and a C3H mouse
mammary carcinoma in vivo**

GÖRAN G. JONSSON



**Department of Oncology
University Hospital, Lund, Sweden**

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To Lenchen, Martin and Per

"The hallmark of science is its state
of incompleteness" (Kurt Salinger)

This thesis is based on the following articles:

- I. Ronald W. Pero, Göran G. Jonsson and Lennart Persson.
Unscheduled DNA synthesis induced by N-acetoxy-2-acetylaminofluorene is not sensitive to regulation by ADP-ribosyl transferase.
Chemical-Biological Interactions 47, 265-275 (1983).
- II. Göran G. Jonsson, Göran Eriksson and Ronald W. Pero.
Effects of γ -radiation and hyperthermia on DNA repair synthesis and the level of NAD^+ in cultural human mononuclear leukocytes.
Radiation Research 97, 97-107 (1984).
- III. Göran G. Jonsson, Göran Eriksson and Ronald W. Pero.
Influence of hyperthermia and γ -radiation on ADP-ribosyl transferase, NAD^+ - and ATP pools in human mononuclear leukocytes.
Radiation Research (1985). In press.
- IV. Göran G. Jonsson, Anders Olsson, Ronald W. Pero, Ann-Kristin Ekholm, Jan Pallon and Sven A.E. Johansson.
Utilization of the PIXE-method for elemental analysis in human mononuclear leukocytes and its application to effects caused by hyperthermia.
Submitted for publication to Journal of Cellular Physiology.
- V. Göran G. Jonsson, Elisabeth Kjellén and Ronald W. Pero.
Nicotinamide as a radiosensitizer of a C3H mouse mammary adenocarcinoma.
Radiotherapy and Oncology 1, 349-353 (1984).

- VI. Göran G. Jonsson, Elisabeth Kjellén, Ronald W. Pero and Robert Cameron.
Radiosensitization effects of nicotinamide on malignant and normal
tissue.

Accepted for publication in Cancer Research February 1985.

In the text these articles will be referred to by their Roman numerals.

ABBREVIATIONS:

ADPRT	adenosine diphosphate ribosyl transferase
AT(D,M)P	adenosine tri (di, mono) phosphate
CAMP	cyclic adenosine monophosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNP	2,4 dinitrophenol
EO	ethylene oxide
[³ H]-dThd	methyl- ³ H-thymidine (tritiated thymidine)
[³ H]-NAD ⁺	adenine- ³ H-nicotinamide adenine dinucleotide
hsps	heat shock proteins
HU	hydroxyurea
I.p.	intra peritoneal
MNU	N-methyl-N-nitrosourea
NA-AAF	N-acetoxy-2-acetylaminofluorene
NAD	nicotinamide adenine dinucleotide
PHA	phytohemagglutinin
PR	phosphoribose
RNA	ribonucleic acid
S.D.	standard deviation
SEM	standard error of the mean
TCA	trichloroacetic acid
UDS	unscheduled DNA synthesis
UV	ultra violet (radiation)
XP	Xeroderma pigmentosum

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11th L.H. Gray Conference on Cellular Repair of Radiation Damage:
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1. INTRODUCTION

The cancer therapy research effort have for many years been directed towards more effective methods for therapy; more lethal for tumor but saving normal tissue and more indulgent for the patient. With regard to radio- and chemotherapy, sensitizing agents have been developed. A large class of radiosensitizers are electron-affinic compounds like O_2 , H_2O_2 , quinone and metronidazoles, which pick up the hydrated electron e_{aq}^- produced in the hydration reaction $\gamma(\text{energy}) + \text{water} \rightleftharpoons OH^\cdot + H^\cdot + e_{aq}^- + \dots$, which then strongly drives the reaction toward the right, resulting in more stabile transient $OH^\cdot$ -radical-ion (Adams and Dewey 1963, Greenstock et al 1974, Vladescu and Apetroae 1983). Unfortunately most electron-affinic agents are difficult to handle or are highly toxic for the patient. Another sensitizing method has been the utilization of supra normal temperatures; hyperthermia in conjugation with radiation and drugs (for reviews see e.g. Cavaliere et al 1967, Dietzel 1975, Dewey et al 1977, Miller et al 1977, Field and Bleehen 1979, Tubiana 1982, Perez 1984).

Other possible ways of sensitizing could be the use of compounds which affect the level of damage and repair introduced by radiation or a cytotoxic agent, i.e. various enzyme inhibitors (Nduka et al 1980, Durkacz et al 1980, Stratford et al 1981).

Besides these biochemical and biophysical sensitizers, there are also "biological response modifiers", which stimulate the immune system like interferon and lymphokines; i.e. agents that inhibit suppressor T-cell function and affect differentiation of tumor cells. Such modifiers may be of importance in the future (see review by Ruddon 1981).

A common component of the mechanism of action of radiosensitizers is that they allow a greater accumulation of DNA damage in irradiated cells. Therefore we have looked for the molecular mechanism of radiosensitizers



to be interfering with the DNA repair mechanisms. γ -radiation is known to kill cells due to the accumulation of DNA strand breaks and adenosine diphosphate ribosyl transferase (ADPRT) is known to be strongly activated by and dependent on DNA strand breaks.

This thesis deals with the effects of two types of radiosensitizers namely hyperthermia and inhibitors of ADPRT especially in relationship to their effects on DNA repair that in turn regulate cytotoxicity in vivo and in vitro.

2. DNA DAMAGE AND REPAIR

Living cells have a complex machinery for replication, synthesis of numerous substances, energy production and for self defense (for review see e.g. Kornberg 1980). Consequently, there are many structures that when disturbed or damaged could be lethal for the cell or interact with its normal function. Knowledge of damage processes, cellular repair processes and about possibilities to interfere with these processes is of greatest importance for understanding of carcinogenesis as well as for developing methods for cancer therapy.

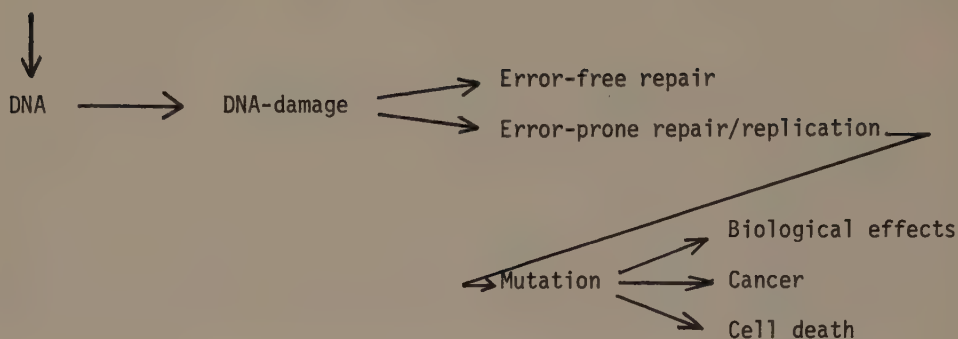
DNA is usually thought to be the most critical target for transformation processes. The double helical DNA molecule, together with a variety of associated proteins, constitute the main components of the chromatin.

The DNA contain the genetic code, as expressed by the codon (i.e. one nucleotide triplet = one amino acid) read in the C-5 to C-3 direction of the four bases adenine, guanine, cytosine and thymine whose integrity is of greatest importance for cell reproduction and cellular metabolism. The number of nucleotide pairs in a single mammalian cell is expected to be about 4×10^9 (haploid set) meaning a total length of double helical DNA to be about 1.5 m (diameter about 20 Å). The chromatin proteins consist of the histones (at least five classes) on which DNA is wound and supercoiled and hundreds of non-histone proteins. Damage to the DNA that prevents accurate base pairing can result in serious consequences for the cell or host organism such as cell death or transformation of the normal cell to a cancer cell with its uncontrolled growth.

γ -radiation

UV-radiation

Certain chemicals



Error-prone repair is due to disturbed proof-reading function and modified polymerization of the DNA (Byrnes et al. 1977), and mutation is due to changes in the sequences of bases.

Different agents induce different kinds of damage to the DNA; γ -radiation mainly create hydroxyl radicals ($\text{OH}^\cdot$) which in turn induce single or double strand breaks, cross links or eventual miscellaneous base damage (Ward 1977, 1981, Painter 1979), UV-radiation produce thymidine dimers (Setlow 1966), the carcinogen N-acetoxy-2-acetylaminofluorene is an alkylator, which covalently binds to the bases (guanine, C-8 position) giving conformational distortions in the DNA helix (Kriek 1974, Westra et al 1976, Miller 1978). Ethylene oxide alkylates DNA (guanine C-7 position) and thereby induces DNA strand breaks (Ehrenberg and Hussain 1981). Typically carcinogenic substances are electrophilic and attack nucleophilic sites in the bases of the DNA.

However, cells whose DNA have been damaged by exposure to genotoxic agents and radiation of the various kinds mentioned above possess a great power to repair this damage. Following damage to the DNA, the cell, as expressed lyrically, "switch on the SOS-response in which several enzymes

function in concert" (Howard-Flanders 1981).

Much of our knowledge about the various steps and enzymes involved in DNA repair has been obtained by the use of mutant strains of bacterial cells which have lost a particular repair function (i.e. E. coli mutants *uvr A-C*, which are deficient of endonuclease) (Grossman et al 1975). Much less is known about repair in mammalian cells, although some information has been obtained from studies of some recessive inherited human disorders associated with defects in the ability of the cells to repair certain kinds of damage to their DNA; Xeroderma pigmentosum, Ataxia telangiectasia and Fanconi's anemia (e.g. review by Setlow 1978).

Patients with Xeroderma pigmentosum (XP) develop UV induced skin cancer with a prevalence among whites at age 20 of almost 100 %. Cells from these patients are also extremely sensitive to nitrogen mustard. Contrarily, these cells repair damage from γ -radiation. Studies with extracts from XP cells have shown that the extracts are capable of removing damage in purified DNA but not in their own chromatin.

The so called excision repair processes involve: recognition of damage with a lesion specific endonuclease, excision of damaged DNA bases by exonuclease, DNA-new-synthesis by polymerase and finally ligation of the new patch to original DNA strand by ligase, Fig. 1 (Cerutti 1974, Kornberg 1980).

The repair enzymes are found among the non-histone proteins associated to the chromatin. The rate limiting event in this excision repair chain seems to be the incision step (Erixon 1980). Arguments for this is the observation that various damage has been found to be additive in normal human cells, even at doses where each agent saturates the repair capacity. This would imply that there are different rate limiting events. Erixon introduces an accessibility factor which together with endonuclease determines the rate of incision and repair (Erixon 1980). This accessibility factor

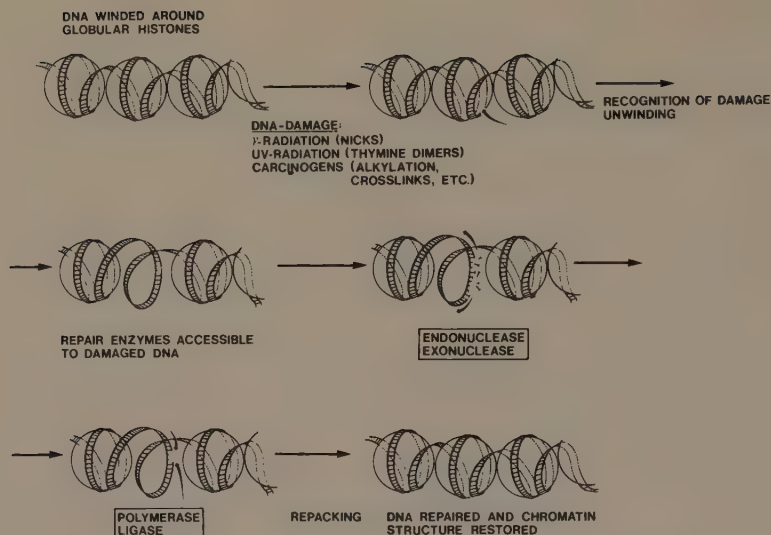


Fig. 1. DNA-repair mechanisms

(figure inspired from a drawing by J.E. Cleaver on the cover of *Cancer Res.* vol. 43, June 1983).

might in normal cells be present in abundance, but the endonucleases are limited in number. XP cells have been found to have a defect in the incision step and could according to Erixon be deficient in the accessibility factor - "the XP-lacking factor", and are then limited in repair capacity for all types of damage requiring excess of endonuclease.

As soon as incision is performed at the site of damage, the DNA supercoiling loop is released and the repair enzymes become accessible to the DNA strand. The loop will stay in this state as long as the nick is present; i.e. until the new synthesized patch is sealed by ligation. Then the superhelical structure is restored.

UV radiation of low wavelength (< 300 nm) give also rise to pyrimidine dimers supposed to be repaired via a DNA-photolyase, which recognize the damage, excises the dimers and restore the DNA structure. This repair mecha-

nism dependent on visible light is called photo enzymatic repair (Setlow 1966). Still another repair mechanism is the so called post replication repair where the DNA replicates with the damage which, however, later might be repaired by genetic recombination (see review by Lehman 1974).

Different types of damage are recognized with different efficiency by the cell and this may result in different lengths of excision (Amacher et al 1977). For γ -radiation and the alkylator ethylene oxide a nick is introduced with only a few nucleotides removed by exonuclease, resulting in "small patch repair" (Reagan and Setlow 1974). Sawada and Okada (1970) report on a direct rejoining mechanism of single-strand breaks of DNA without preceeding repair synthesis. Other genotoxic agents like N-acetoxy-2-acetylaminofluorene (NA-AAF) and also UV-radiation involve a 100 nucleotides and result in so called "large patch repair" (Cleaver 1973, Reagan and Setlow 1974). Bad "recognition of damage" and large patch repair are repaired more slowly than easily recognized and short patch repair. This is also clearly demonstrated in Fig. 2, which shows repair as measured by

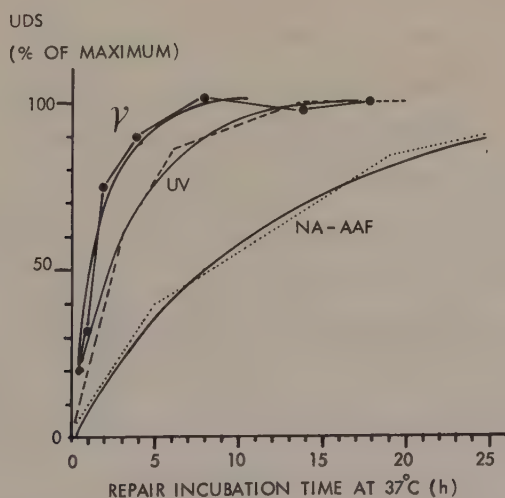


Fig. 2. Unscheduled DNA synthesis versus time of incubation at 37°C following damage by γ - (Jonsson 1982), UV-radiation and NA-AAF (Pero and Østlund 1980).

unscheduled DNA synthesis (UDS), following exposure of human mononuclear leukocytes to γ -radiation, UV-radiation and NA-AAF versus repair incubation time (t).

The solid drawn curves are fits to the experimental data of the formula $UDS (\%) = 100 [1 - \exp(-kt)]$, where $k = \ln 2 / t_{50\%}$ and $t_{50\%}$ the time for repair to 50% of maximum. For γ -radiation, UV-radiation and NA-AAF the $t_{50\%}$ -values are about 1, 2 and 8 hours respectively.

However, repair kinetics are probably more complex than expressed by this simple relation. For γ -radiation on human lymphocytes a fast and a slow component of repair has been identified. The fast step is supposed to be fulfilled within about 1 h, the slow within about 10 h (Spiegler and Norman 1969).

DNA repair processes are extremely complex and only some fragments are understood. Almost nothing is known about regulatory functions, although there is evidence that phosphorylation, methylation, acetylation and poly ADP-ribosylation have roles in DNA replication and repair (Shall et al 1977, Lindahl 1982).

It has also been suggested that cAMP and drugs that elevate intracellular cAMP by activating adenylate cyclase or inhibiting phosphodiesterase play roles in cell proliferation, differentiation and repair (Ryan and Heidrich 1968, Pastan et al 1975, Tolmac et al 1977, 1980).

Modification by poly ADP-ribosylation is remarkable because of the big size and highly negative charge of the polymer and thus might cause distortions in the chromatin and in turn facilitate repair processes (Lindahl 1982).

3. ROLE OF HYPERTHERMIA

Hyperthermia is defined as temperature exceeding the physiological body temperature - in this thesis the range 37-46°C was studied.

Hyperthermia as a therapeutic agent by itself against cancer was suspected already more than 100 years ago. The earliest reports dealt with cancer patients who accidentally got repeated attacks of erysipelas and thus high fever where one surprisingly in some cases found spontaneous regression of the tumors (Busch 1866, Coley 1893, Rohdenburg 1918). Deliberated fever induced mainly by erysipelas, but also by malaria, was then used by many clinicians to treat various types of tumors (Warren 1935, Braunstein 1929, Nauts et al 1953). Nauts and collaborators (1953) made an analysis of protocols of 30 cancer patients treated with highly pyrogenic toxin extracted from Streptococcus erysipelatis and Bacillus prodigiosus (Coley's toxin). 25 of these patients were alive and free of disease more than 10 years afterwards! The effects induced by pyrogenes were of course very difficult to predict and control. Passive total-body heating was tried, which, however, also gave undesired effects on homeostasis and on the endocrine and immune systems. This approach is nonetheless still being utilized. Therefore, the interest was directed towards the use of local hyperthermia. The first experiment with local heating was in fact done already in 1898 by Westermarck in the treatment of cervix cancer (see review by Cavaliere et al 1967).

Hyperthermia as a radiosensitizing agent was suggested by Schmidt 1909. This method has since then been explored by many authors (see e.g. Overgaard 1935, Müller 1912) and is now used in many hospitals (U et al 1980, Perez et al 1981, Manning et al 1982, Lindholm et al 1982, Arcangeli et al 1983, Tubiana 1982, Perez 1984, Luk et al 1984).

Similarly, hyperthermia has been tried together with cytostatics

with promising results (Woodhall et al 1960, Giovanella et al 1970, Stehlin et al 1975, Krementz et al 1977, Hahn 1979, Dethlefsen and Dewey 1982, Hugander 1983).

Today it is possible with refined techniques to study both experimental tumors and cells growing in culture, and it is equally possible to investigate effects of various treatments at the macroscopic as well as the molecular level.

Many experiments indicate that there is a synergistic effect of radiation and hyperthermia; i.e. the cytotoxicity of the combined treatment is larger than the sum of toxicities of hyperthermia and radiation alone (Ben-Hur et al 1972, Overgaard and Overgaard 1974, Dewey et al 1977, Sapareto et al 1978, Raaphorst et al 1979). It is interesting to note that cells are most sensitive to hyperthermia treatment in late S-phase where the cells are most radio-resistant. Dividing cells are most radio-sensitive at or close to mitosis (Westra and Dewey 1971, Dewey et al 1978, Mittler 1981).

One important question is if there is any selectivity of hyperthermia on tumor tissue when compared to normal tissue. There are confusing discrepancies regarding this question with papers indicating that tumor cells should be more sensitive to heat than normal cells (Overgaard 1976, Kase and Hahn 1975, Giovanella et al 1976), but the opposite has also been proposed (Harisiadis et al 1977).

Of course, when increasing the temperature high enough all cells are killed, although some tissue types (e.g. intestine) are more sensitive than others (e.g. skin). Temperature (T) and the heating time (t) for any biological effect are in the temperature region 41-47°C connected via an approximately exponential relation; $t \sim \exp(k \cdot T)$, where the constant k has been found to be typically about -0.7°C^{-1} (Henle and Dethlefsen 1980). This means that when increasing the temperature with 1°C , the heating time can be re-

duced to about half to give the same biologic effect. Lymphocytes seem to be more sensitive to thermal injury than many other cell types (Agarwal et al 1983). Lymphocytes exposed to 42°C for 4 hours lowered thymidine incorporation at early times after PHA stimulation but the cells recovered when returning to 37°C . However, at 43°C exposure for even 1 hour caused irreversible damage. The data by Henle and Dethlefsen (1980), indicate that there is no greater sensitivity of tumor cells than of normal tissue. However, the authors point out that a selective effect of combined treatment on tumor tissue can be achieved if environmental factors, such as the already low tumor pH is taken into account and by utilization of radiation fractionation procedures. The importance of host response in the destruction of tumor was also stated by Dickson and Calderwood (1980). High temperatures, which by itself cause damage, given before radiation was found to induce greater cytotoxicity than heat after radiation (Sapareto et al 1978). This was proposed to be due to inhibition of repair of radiation damage. Hyperthermia given in immediate connection to radiation selectively reduces survival of radio resistant S-phase cells.

The background of the hyperthermia effect is by no means fully understood. There are probably effects on micro circulation on the tissue level affecting oxygen tension, pH and nutrition, on immune system, on the sub-cellular level and on the molecular level. There is also a possibility that hyperthermia alone can produce a small number of DNA strand breaks at high heat doses (Juarez-Salines et al 1984).

When plotting the inverse of the 37% dose slope parameter of survival curves versus the inverse of the hyperthermia temperature ($^{\circ}\text{K}^{-1}$) in a so called Arrhenius representation, one typically finds a change of slope at about 43°C for the majority of cell types studied (see analysis of various data by Henle and Dethlefsen, 1980). From the slopes the inactivation energies associated with the decreasing survival can be deduced. For heat treat-

ments in the range below 43°C the inactivation energy is about 300-400 kcal/mole. In the temperature range $43-46^{\circ}\text{C}$ the inactivation energy is typically between 100 and 150 kcal/mole. The observed inactivation energy at high temperatures is about of the same size as inactivation energies of several enzymes and proteins and suggest that heat inactivation could be due to denaturation of a critical protein (Dewey et al 1977). Different inactivation energies might indicate different mechanisms of cell killing i.e. different targets for cytotoxicity in the two temperature regions (Dikomey 1981). In addition, different effects on the proliferative capacity and/or repair have also been observed in the combined treatment of γ -radiation with hyperthermia at 42 and 45°C , respectively (Dikomey 1981). Analogous to damage by radiation, Henle and Dethlefsen (1980) and Roti Roti and Henle (1980) postulate that below and above 43°C the inactivation energy could be associated with a dominance of multiple-hit events and single-hit lethal events, respectively, although no such mechanisms have been identified yet.

Furthermore there is the concept of thermotolerance meaning that mammalian cells, at least when grown in vitro, can develop tolerance to thermal damage after heat conditioning (see review Henle and Dethlefsen 1978). At temperatures below 43°C survival of cells decrease rapidly with increasing temperature followed by onset of tolerance after a few hours of heating. At temperatures above 43°C thermotolerance is not observed. Li et al (1982) show that the change of slope in most Arrhenius plots probably results from development of thermotolerance at temperatures below 43°C and that there then should be only one mechanism responsible for inducing cell death in the whole temperature range ($40-47^{\circ}\text{C}$).

It is important to know about all these effects to be able to optimize conditions for combined treatment of hyperthermia with radiation or with chemotherapy; when and how much heat should be given, role of frac-

tionation protocols, etc.

Circulatory effects. At temperatures above 37°C but below 43°C the tissue response is vasodilatation and increased micro blood flow which means increased oxygen tension (Bisher et al 1980). Since γ -radiation interacts with tissues mainly via production of free hydroxyl radicals, then increased oxygen tension means more stabile ion-radicals and more damage is induced. Above 43°C vasoconstriction takes place, resulting in cessation of blood flow. This give reduced oxygen tension and reduction of the already low tumor pH, which by itself, may be more lethal for tumor than for normal tissues (Bisher et al 1980, Vaupel et al 1980, Song 1982).

Effects on the immune system. It was suggested by Crile (1963) that the spontaneous regression of tumors noted to follow infections might not be due to hyperthermia per se, but caused by some components involved in the inflammatory reaction. Dickson and Shah (1980) proposed that the remarkable cures of patients treated with pyrogenes (see page 9) were caused by stimulation of immune system. Micro organisms or their products inducing the inflammatory reaction could have a direct destructive effect on the cancer cells, possibly even rending them immunogenic. In animal systems and whole-body heating, hyperthermia has been found to increase the incidence of metastasis (Yerushalmi 1976, Dickson and Ellis 1976), which was assumed by Dickson and Shah (1980) to be due to direct damage of the lymphoid tissue of the experimental animals. Consistent with this is the finding that hyperthermia suppresses human Natural Killer (NK) cell activity probably more than other lymphocyte sub classes (Azocar et al 1982, Kalland and Dahlqvist 1983). Both recognition of target cell as well as the NK-mediated lytic processes are affected (Kalland and Dahlqvist 1983).

However, following local heating of tumors, changes in the cell membranes and also products from heat killed cells have been suggested to indeed increase immunogenicity of tumor cells (Mondovi et al 1972, Pantazatos et al 1978). It has also been reported that distant metastases disappear after giving local hyperthermia of the primary tumor in animal model systems (Schechter et al 1978, Shah and Dickson 1978).

There are only a few studies of the immune system for hyperthermic treatments of human tumors in vivo. De Horatius et al (1977) found in patients with malignant diseases exposed to whole-body hyperthermia (42°C for 2 hours) an increase in the number of T-lymphocytes and a decrease in complement activity. In a similar study Koga et al (1983) noted a decrease of the hosts immune response i.e. decreased lymphocyte blastogenesis, lymphocyte rosette formation, Ig G and C3c component, although these parameters returned to the pretreatment level about a week after hyperthermia treatment. Combined local hyperthermia and γ -radiation of cutaneous tumors have also been found to have a reversible suppressive effect on NK cells (Kalland 1985).

Subcellular level

(a) Cell membranes. Hyperthermia may affect lipid composition, fluidity, protein and ion permeability of the cell membrane. Coss et al (1979) found with electron microscopy large evaginations at the plasma membrane indicating weakening of the membrane. Changes in the surface morphology probably affect surface antigens and other receptors. When cells grow at high temperatures, their unsaturated fatty acid content declines and more saturated fatty acids are incorporated into the phospholipids and thermotolerance increases (Mc Elhaney and Souza 1976). It has been reported (Yatvin 1977) that the sensitivity of bacteria (E. coli) to hyperthermia depends on the amount of unsaturated fatty acids in the membranes; i.e. the higher the unsaturated fatty acid content

the higher the heat sensitivity leading to membrane disorganisation and cell death. There is also a strong correlation between the heat sensitivity and decreased cholesterol content of cell membranes in different mammalian cell lines (Cress et al 1978). The cholesterol concentration decreases with duration of hyperthermic treatment as survival decrease. Dynamics of lipids, as measured by electron spin label mobility, is similarly dependent on lipid composition and temperature (King et al 1977). Transport systems for purines (Harms-Ringdahl 1984) and amino acids (Kwock et al 1978) are susceptible to heat exposure and depend on lipid composition. The amino acid transport kinetic parameter $V_{\max}$ (rate of turnover) is reduced, but carrier affinity K_m is unaffected, which indicates that hyperthermia either reduces the number of carriers in the membrane and/or the rate of transport across the membrane (Kwock et al 1978). Furthermore, $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity is temperature sensitive (Wisnieski et al 1974), which in turn could be one explanation to the finding that the intracellular K is lost and Na gained irreversibly as temperature is increased above 42°C (Yi 1979, Yi et al 1983a).

(b) Heat shock proteins and thermotolerance. Following a wide variety of environmental stresses, cells of almost all organisms have been shown to within minutes synthesize a family of proteins (polypeptides) with well defined molecular weights named "heat shock proteins" hsp's (Ashburner and Bonner 1979, Vincent and Tanguay 1979, Currie and White 1981, Rubin et al 1982, Schlesinger et al 1982, Tomasovic et al 1983, Li 1983). The stress could be heat, chronic hypoxia, ethanol, lidocaine, or energy deprivation and yet the same proteins could be formed (Ashburner and Bonner 1979, Li 1983). Stress affects the genome by both initiating and repressing the synthesis of specific RNA at several different levels. This results in translation of a set of specific polypeptides while other macromolecular synthetic processes

are shut down (Schlesinger et al 1982, Ashburger and Bonner 1979). The role of these hsp's is not clear, but it seems as if these proteins protect the cell against a still further lethal temperature challenge or other environmental stresses; i.e. thermotolerance has developed (Li and Werb 1982, Li 1983). The onset of thermal resistance was found to take place earlier with slow temperature increases than with rapid transient temperature changes (Tomasovic et al 1983). Rubin and collaborators (1982) have suggested that one or more of the hsp's are protein kinases which could modify post translational proteins by phosphorylation. Schlesinger et al (1982) postulate that at least some of the hsp's can modify nuclear and cytoplasmatic cytoskeletons so that the metabolic apparatus is protected from further stress.

(c) Effects on lysosomes. Various studies have given evidence that lysosomal activity may be involved in hyperthermia killing. Overgaard (1976) found an increased number of lysosomes in transplanted mouse mammary carcinoma within the first hours after treatment with 42.5°C for 30 min. Many authors have, with histochemical staining methods, in various tissues and cells, found at least temporary increased activity of the lysosomal enzymes acid phosphatase and β -glucuronidase (Hume et al 1978, Barratt and Wills 1979, Myers et al 1981). Myers et al (1981), using cartilage of baby rat tails, also studied the effect of γ -radiation and found no effect on β -glucuronidase activity. However, the combined treatment of γ -radiation (4 Gy) given immediately after heat treatment (44°C for 15 min) caused a greater and more prolonged activity of the enzyme than heat alone. It is of interest to note that γ -radiation given immediately before heat treatment gave no such synergistic effect. Contrary to these findings, Tamulevicius and Streffer (1983) found no effect of hyperthermia on these enzyme activities using biochemical techniques, even at temperatures of 43°C for 45 min. This confusing

discrepancy was explained to be due to the different experimental techniques used! Magun (1981) reported that lysosomal function, as measured by the ability to degrade epidermal growth factor in cultured rat fibroblasts, was considerably inhibited by hyperthermia of 45°C for 30 min. The lysosomal function recovered only partially, even up to 14 h after heat exposure.

(d) Hyperthermia and DNA damage and repair. The first quantitative studies were done by Ben-Hur et al (1972, 1974). They reported that radiation response is enhanced by hyperthermia and appear to result from an inhibition of the repair of sub-lethal damage as well as an enhancement of lethal damage. This finding has been later confirmed by many authors (Dewey et al 1978, Warters and Roti Roti 1979, 1981, Lunec et al 1981, Clark et al 1981, Mills and Meyn 1981, Spiro et al 1982, 1983, Davis et al 1983 etc.).

Warters and Roti Roti (1979) suggested that the sub-cellular mechanism for heat-induced inhibition of excision repair capacity is damage to chromatin rather than denaturation of the enzymes involved. This suggestion was based on the finding that excision enzymes from heated cells (45°C for 30 min) were able to recognize γ -ray induced damage of unheated chromatin. The idea was also supported by Clark et al (1981) who concluded that the reduction in rates of rejoining of DNA strand breaks involved structural changes in DNA (i.e. the supercoiling). The reduced activity of repair enzymes was supposed to result from decreased enzyme-substrate affinity rather than denaturation of enzyme molecules. Hyperthermia above 42°C increases the relative amount of proteins associated with DNA and there will also be a higher amount of non-histone proteins bound to the DNA (Clark et al 1981, Mills and Meyn 1981, Wheeler and Warters 1982). Lunec et al (1981) found that subsequent rate and extent of DNA strand break repair are significantly reduced by pre-irradiation heat treatment (44°C for 30 min). However, this effect was abolished when an incubation period of 1 h at 37°C was introduced between

heat treatment and irradiation. Obviously the recovery from hyperthermia treatment is quite rapid. Mills and Meyn (1981) found both initially increased amount of DNA strand breaks as well as substantial inhibition of DNA strand rejoining after combined treatment of hyperthermia and radiation. Heat radiosensitization has also been found to correlate with loss of intracellular DNA polymerase- β activity (Spiro et al. 1982, 1983).

(e) Hyperthermia and energy production. DNA repair requires energy in the form of ATP (Waldstein et al 1974, Kaufmann et al 1982, Dresler and Lieberman 1983). Heat stress has been found to reduce cellular ATP (Francesconi and Mager 1979, Ohyama and Yamada 1980, Lunec and Cresswell 1983). However, ATP reduction following treatment with hyperthermia probably does not take place in all cell types. Henle et al (1984) found no significant depletion of ATP in HeLa cells given hyperthermia, neither at 41.5 nor at 45°C, although there is a strong decrease in survival at the highest temperature (Henle and Dethlefsen 1978). Heat has an inhibitory effect on cell respiration (Muckle and Dickson 1971, Cavaliere et al 1967, Mondovi et al 1969), which in turn also may affect ATP production. Damaged energy production mechanisms could explain the decreased oxygen uptake found at temperatures above 42°C (Muckle and Dickson 1971, Dickson and Shah 1972). At high glucose levels, hyperthermia at 44°C has been found to increase the anaerobic utilization by 130% above the level at 38°C (von Ardenne and Reitnauer 1966). Quite logically hyperthermia has been reported to enhance killing of glucose-deprived hypoxic cells with much smaller effects on glucose-deprived cells under aerobic conditions (Kim et al 1978, Song et al 1979, Laval and Michel 1982). However, when there is a demand of energy, which is the case after DNA damage, the energy production might be critical.

Combinations of the mechanisms described above may contribute to the increased cytotoxicity of the combined treatment of hyperthermia and radiation.

Damage to the plasma membrane and the nuclear membrane can cause changes in intracellular ion contents, which in turn can alter the activity of repair enzymes (Spiro 1983). Damaged lysosomes release proteases, which also may affect enzymes e.g. polymerase- β . Hyperthermia has an inhibitory effect on RNA synthesis which in turn may affect protein synthesis and DNA repair (see paragraph about heat shock proteins).

In conclusion, agents that allow greater accumulation of DNA strand breaks could act as a sensitizer when combined with γ -radiation.

The results presented in this thesis indicate that hyperthermia and ADPRT inhibitors could both behave like such agents!

4. ROLE OF ADENOSINE DIPHOSPHATE RIBOSYL TRANSFERASE

A substantial amount of research has been directed toward investigating the possible role of poly ADP-ribosylation in cell proliferation and DNA excision repair (see reviews by Hilz and Stone 1976, Hayaishi and Ueda 1977, Purnell et al 1980, Miwa et al 1983).

Adenosine diphosphate ribosyl transferase - ADPRT, also named poly (ADP-ribose) synthetase or poly (ADP-ribose) polymerase, is a nuclear enzyme, which covalently attaches ADP-ribose moieties derived from NAD^+ to chromatin proteins resulting in a homopolymer of ADP-ribose residues linked by C-1, C-2 ribose-ribose bonds, Fig. 3. The enzyme was discovered by Chambon et al (1963) who, however, believed that ATP was the immediate substrate. This conclusion was supported by Fujimura et al (1967). The same year Nishizuka et al (1967) found that NAD^+ , rather than ATP, was the substrate for the enzyme and that ATP was not incorporated until it was converted to NAD^+ . They also found the concomitant release of nicotinamide. Whether the enzyme catalyzes the transfer of an ADP-ribose residue to the acceptor protein is not clear although both initiation and elongation has been proposed (Hilz and Stone 1976).

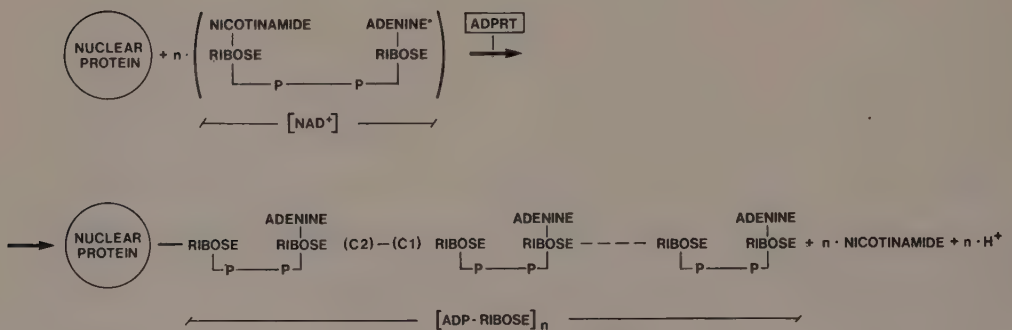


Fig. 3. Synthesis of the poly (ADP-ribose)-protein complex

ADPRT has been purified about 10^4 fold from pig thymus and found to have a molecular weight of about 60.000 (Tsopanakis et al 1978). The enzyme is dependent on DNA and histones for activity and is strongly stimulated by DNA strand breaks, thereby causing accumulation of poly (ADP-ribose) and concomitant consumption of the cellular NAD^+ pool. NAD^+ is not only substrate for ADPRT, it participates in numerous enzymatically catalyzed oxidation-reduction reactions. Maintaining a high level of NAD^+ is therefore of great importance for cellular life. There are in mammalian cells essentially three different precursors for NAD^+ biosynthesis; the amino acid tryptophan and the vitamins nicotinamide and nicotinic acid (Preiss and Handler 1958, Nishiguka and Hayaishi 1963, Ijichi et al 1966, Hillyard et al 1973, Jacobson et al 1979, Foster and Moat 1980, Johnson 1980). The biosynthesis of NAD^+ is dependent on ATP.

Recently, it has been reported that the $(\text{ADP-ribose})_n$ -polymer both in vitro and in vivo has a branched structure (Miwa et al 1981, Juarez-Salinas et al 1982, 1983). The number of ADP-ribose moieties has been found to vary from only a few up to about 200.

The polymer is degraded by at least two classes of enzymes; (i) phosphodiesterases, catalyzing the hydrolysis of the pyrophosphate bonds with the release of PR-AMP (Futai et al 1967) and (ii) poly (ADP-ribose) glycohydrolases, which split the glycosidic C-1, C-2 ribose bounds producing ADP-ribose residues (Miwa and Sugimura 1971, Hilz and Stone 1976).

The nature of the bounds linking poly (ADP-ribose) to acceptor proteins is not known although ester glycosidic bounds have been suggested (Purnell et al 1980).

A variety of proteins have been suggested to serve as acceptors of the ADP-ribose tail; (i) Ca^{++} -, Mg^{++} - dependent endonuclease, with concomitant inhibition of the enzyme activity (Yoshihara et al 1975, Nomura et al 1981), (ii) ligase which either needs direct ADP-ribosylation for activity (Creissen

and Shall 1982) or by counteracting inhibition of ligase by histones (Ohashi et al 1983) thus facilitating rejoining of broken DNA strands, (iii) histones (Tanuma et al 1977), (iv) RNA polymerases, or automodification of ADPRT itself (Hilz and Stone 1976, Yosihara et al 1977, Ohashi et al 1983).

The level of poly ADP-ribosylation has been found to vary with the cell cycle. For nuclear systems the level of poly (ADP-ribose) peaks in the G2-M phases and is lowest in the S phase (Hilz and Stone 1976). Similar results have been found for partially purified enzyme preparations. These results indicate that poly-ADP-ribosylation of nuclear proteins may play a fundamental role during chromosome condensation (Hilz and Stone 1976).

The role of ADPRT in cells, i.e. of poly (ADP-ribose) or ADP-ribosylation of chromosomal proteins is not fully understood, although there have been convincing data reported of its involvement in DNA repair, cellular differentiation, gene expression and perhaps in carcinogenesis (Miwa et al 1983).

These different functions of ADPRT are probably due to various subclasses of poly (ADP-ribose)-protein conjugates; e.g. mono ADP-ribosylated proteins are associated with cell proliferation and differentiation, while poly ADP-ribosylated protein conjugates are restricted to the nucleus and associated with DNA strand breaks and repair (Hilz et al 1978, Wielckens et al 1982a).

The role of NAD^+ as a cosubstrate in regulating ADPRT activity in cell division and differentiation is demonstrated by the finding that NAD^+ levels are significantly lower in dividing cells than in non-dividing cells. A variety of proliferating tissues such as tumors, fetal tissue and regenerating liver also contain lower NAD^+ levels than low proliferating tissues (Jacobson and Jacobson 1976). Since NAD^+ is a co-substrate for ADPRT then quite logically its activity has been found to be higher in regenerating liver, in hepatoma cells, leukemic cells and in mitogenic stimulated lymphocytes than in normal cells (Kidwell and Mage 1976). Berger et al (1978a)

found a mean basal level of ADPRT activity in chronic lymphocytic leukemia lymphocytes to be 2,5 times higher than the activity in lymphocytes from healthy blood donors. When DNase treated the leukemic cells got 2,9 times higher ADPRT activity than normal lymphocytes treated similarly. The authors argue that the high ADPRT activity could be due to the presence of immature proliferating lymphocytes in the peripheral blood from patients with leukemia or the presence of leukemic cells with damaged or disordered DNA.

The possible connection of ADPRT to NAD^+ pools in cellular differentiation have been used to explain the congenital abnormalities and decreased ability to recover from DNA damage that is characteristic of cells from patients with Fanconi's anemia (Berger et al 1982a). Cells from these patients show more spontaneous chromosome aberrations and are also much more sensitive to certain carcinogens than normal cells which indicate some defect in DNA repair. Lymphocytes derived from such patients were shown to activate ADPRT following UV irradiation as well as following treatment with N-methyl-N'-nitro-N-nitrosoguanidine, but the cells were found to have very low NAD^+ levels and after damage the NAD^+ pools dropped to values much lower than in similarly treated cells from normal donors. This may severely impair the ability of Fanconi's anemia cells to maintain their energy metabolism during the period required for normal DNA repair (Berger et al 1982a).

Role of ADPRT in DNA damage and repair. DNA-damaging agents which induce strand breaks and thereby stimulate the enzyme cause a rapid but temporary depletion of the cellular NAD^+ pools (e.g. Roitt 1956, Campagnari et al 1966, Skidmore et al 1979, Jacobson et al 1980, Rankin et al 1980, Busbee et al 1980, Durkacz et al 1980, Wielckens et al 1982b, Sims et al 1982, Jacobson et al 1983, 1984a, Ben-Hur and Elkind 1984). In order to keep poly (ADP-ribose)

residues high during DNA repair, a high turnover of poly (ADP-ribose) and a high capacity of NAD^+ resynthesis are required. The activity of fully stimulated ADPRT in human lymphocytes has been found to have the capacity to consume the total cellular NAD^+ within minutes (Wielckens et al 1980)!

The requirement of nicked DNA for increased ADPRT activity has been shown by Berger et al (1980b) in an elegant experiment on lymphocytes from patients with XP. These XP cells were found not to respond with increased ADPRT activity following UV irradiation. This defect was suggested to be secondary to the failure of these cells to create DNA strand breaks in the region of UV-induced DNA damage (i.e. excise the thymidine dimers - see page 6). The XP cells exhibit normal increased poly (ADP-ribose) synthesis following treatment with N-methyl-N'-nitro-N-nitrosoguanidine. Similar results were found by McCurry and Jacobsson (1981) when they also studied NAD^+ pools in XP cells.

It should, however, be noted that there are inconsistencies in the data dealing with the role of ADP-ribosylation in DNA synthesis, where some papers report increased coincident incorporation of ^3H -labeled thymidine into damaged DNA, and other papers report decreased incorporation or even no effect at all (Purnell et al 1980). Most of these experiments were done on isolated cell nuclei because the substrate NAD^+ does not penetrate intact cells. Berger et al (1978b) and Halldorsson et al (1978) have suggested that permeabilized cells are better in reflecting the situation in vivo because the chromatin then is less damaged than in isolated nuclei.

Jacobson and collaborators (1984b) worked with intact cells utilizing immobilized boronate resins which absorb both polymeric and monomeric ADP-ribose from the cell. The adenine-containing compounds are then transformed to highly fluorescent derivatives which can be determined at the pmol level. It was then found that DNA damaging agents cause a rapid conversion of NAD^+ to poly (ADP-ribose) also in vivo.

Another approach using intact cells for studying the role of ADP-ribosylation is the use of inhibitors to the enzyme. There are many compounds that can inhibit the ADPRT activity; NAD-analogs, nicotinamide analogs (nicotinamide, nicotinic acid, benzamide and other aromatic amides), methylxanthines and thymidine and their analogs (Hilz and Stone 1976, Althaus 1982, Sims et al 1982). The drop in cellular NAD^+ following treatment of cells with damaging agents is prevented by these inhibitors (Skidmore et al 1979, Jacobson et al 1980, Busbee et al 1980, Durkacz et al 1980, Wielckens et al 1982b, Sims et al 1982, Jacobson et al 1983, 1984a). The effectiveness of the inhibitors in blocking the consumption of the NAD^+ in intact cells has been found to be roughly related to their apparent K_i -values (K_i = inhibitor constant in the Michaelis-Menten formalism) in isolated nuclei (Shall et al 1977). A low K_i -value means effective inhibitor ($K_i[\text{nicotinamide}] = 11\text{--}15 \mu\text{M}$), and a high K_i -value defines a weak inhibitor ($K_i[\text{caffeine}] = 200\text{--}275 \mu\text{M}$). Benzamide is found to be the most effective inhibitor with a 50% reduction of cell ADPRT activity already at a concentration of about $5 \times 10^{-5} \text{ M}$ (Oikawa et al 1980). Nicotinamide and theophylline need a concentration in the order of 10^{-3} M (Jonsson 1982).

It is reasonable to believe that inhibitors to ADPRT could influence the ability of DNA repair and thereby increase the cytotoxicity of DNA damaging agents. This has also been found in many experiments. For example:

- (1) Calcutt et al (1970) reported a radiosensitizing effect of nicotinamide (400 $\mu\text{g/kg}$) on transplanted sarcoma S.180 tumors in BALB/c mice.
- (2) Smulson et al (1977) found that nicotinamide (500 $\mu\text{g/kg}$) increased the enhancement of survival of mice injected with L1210 mouse leukemia lymphoblast cells treated with various doses of N-methyl-N-nitrosourea, compared to animals given N-methyl-N-nitrosourea alone. They also reported that nicotinamide (100 mM) increased

- degradation of DNA from HeLa nuclei damaged with the same carcinogen.
- (3) Shall et al (1977) showed that co-administration of 5-methyl-nicotinamide or theophylline considerably increased the cytotoxicity of streptozotocin in L5178 Y cells.
 - (4) Nduka et al (1980) found that 2 mM 5-methyl-nicotinamide dramatically enhanced the cytotoxicity of MNU and to a lesser extent also of γ -radiation in L1210 cells. Also methylxanthines and thymidine enhanced the cytotoxicity of the carcinogen.
 - (5) Durkacz et al (1980) reported that 2 mM 3-aminobenzamide greatly increased the cytotoxicity of dimethyl sulphate in L1210 cells.
 - (6) Theophylline and 1-3-Bis (2-chloroethyl)-1-nitrosourea act synergistically to kill L1210 leukemia cells in vivo (De Wys and Bathina 1980)
 - (7) Zwelling et al (1982) reported that 10 mM 5-methyl-nicotinamide and 5 mM 3-aminobenzamide slow the re-sealing of γ -ray induced DNA strand breaks in L1210 cells.
 - (8) Barra et al (1982) concluded that 20 mM nicotinamide and isonicotinamide both can inhibit unscheduled DNA synthesis in HTC cells as well as in hepatocytes incubated with bleomycin, although they had only little effect on the DNA structure.
 - (9) The anti-tumor effect of bleomycin was strongly potentiated by ADPRT inhibitors in intraperitoneally implanted Ehrlich ascites carcinoma cells in ICR mice, more strongly with benzamide and to a lesser extent by nicotinamide (Sakamoto et al 1983, Miwa et al 1983).
 - (10) When combined with MNU, 2 mM aminobenzamide almost completely reduced the cloning efficiency of CHO cells (Kidwell and Purnell 1983). Furthermore, ADPRT inhibitors alone in mM-concentrations was shown to reduce cell growth for CHO and HeLa cells.
 - (11) Tolmach and his group (Beetham and Tolmach 1984) have looked at the action of caffeine on X-irradiated HeLa cells. 10 mM caffeine was

present for 6 hrs after irradiation of the cells with 2 Gy X-rays. The authors conclude that X-rays do not sensitize the cells to caffeine, but rather that caffeine enhances the expression of potentially lethal radiation-induced damage.

- (12) Heartlein and Preston (1985) found that there is an enhancing effect of 3-aminobenzamide (5 mM) on X-ray induced chromosome aberration frequency (about 2-fold) in human lymphocytes, although only when pre-treated with PHA (and in G₁ phase of the cell cycle). The data demonstrate the importance of cycling cells for ADPRT inhibitors to exert their enhancing effect. No such potentiating effect was found in lymphocytes irradiated with fission neutrons in the presence of enzyme inhibitor.

There are, however, experiments showing the direct opposite effects of ADPRT inhibitors, i.e. enhanced viability or no cytotoxicity:

- (1) Bohr and Klenow (1981) reported that 3-aminobenzamide stimulate DNA repair in human lymphocytes but inhibit repair in L1210 cells.
- (2) James and Lehman (1982) found no effect of 5 mM 3-aminobenzamide on viability of UV irradiated diploid fibroblast cells.
- (3) For mammary epithelial cells 3 mM aminobenzamide have been found to stimulate growth and increase collagen synthesis (Kidwell and Purnell 1983).
- (4) Kol and Ben-Hur (1983) conclude from their experiments on mitogen-stimulated human lymphocytes that nicotinamide protect the cells from damage by γ - and UV-radiation.

Thus it seems as if the response of cells to various ADPRT inhibitors vary with cell type, growing conditions and treatment. There are also possibilities that these inhibitors could affect other vital cellular activities than blocking the ADPRT acitivity.

In conclusion we note that ADP-ribosylation of nuclear proteins is by no means well understood, and that ADP-ribosylation possibly is involved in many different cellular events. Many data indicate that ADP-ribosylation has a role in DNA repair and that inhibitors of ADPRT do increase the cytotoxicity of DNA damaging agents. There is some evidence that ADPRT activity might not be directly necessary for repair replication (Nolan and Kidwell 1982, Jacobson et al 1983). However, ADP-ribosylation is certainly somehow involved in DNA damage and repair, either (i) in the initial step by affecting chromatin structure and incision, (ii) in later steps by facilitating ligase activity and re-sealing or (iii) in by-passing some regulatory function associated with repair. As pointed out by States and Janekidevi (1983) and also by Juarez-Salinas et al (1984) it is possible that poly (ADP-ribose) synthesis is part of a general response to environmental stress making the cell more resistant to further damage. The role of poly ADP-ribosylation (due to big size and large negative charge) could very well be in affecting chromatin higher order structure. Inhibition of ADPRT then may affect recovery from damage; i.e. via disturbing excision repair, control of DNA replication or cause a cell cycle hold.

Since many of the ADPRT inhibitors are not very toxic and easy to administrate (like nicotinamide) these substances could have a role as sensitizers in cancer therapy.

5. AIMS OF THIS THESIS

- (i) To study how various DNA damaging agents, which induce different rates of UDS, differ in capacity to stimulate ADPRT in cultured human mononuclear leukocytes.
- (ii) To investigate effects of ADPRT inhibitors (nicotinamide and benzamide) on UDS following various types of DNA damage.
- (iii) To look for effects of hyperthermia on UDS and nucleotides essential for DNA repair.
- (iv) To investigate the role of ADPRT in hyperthermia as well as the role of inhibitors of ADPRT as mechanisms of radiosensitization.
- (v) To look at intracellular levels of some ions in human mononuclear leukocytes following exposure of hyperthermia. Changes in intracellular ionic levels are namely of greatest importance for regulating pH, cell volume, DNA repair enzymes etc. The method of Proton Induced X-ray Emission is used for determination of ionic concentrations and the validity of this method for multielemental analysis in biological systems is stressed.
- (vi) To investigate effects of one ADPRT inhibitor (nicotinamide) as a radiosensitizer in vivo, utilizing transplanted C3H mouse mammary adenocarcinomas as model system. Effects on normal tissue is also studied in a tail stunting experiment on BALB/CA mice.

From the data obtained one model for the effects of hyperthermia on mononuclear leukocytes is discussed.

6. EXPERIMENTAL DETAILS

A. IN VITRO experiments using human mononuclear leukocytes (papers I - IV)

(1) Cell cultures. Human venous blood was collected from apparently healthy donors. The platelet rich plasma was removed by centrifugation at $100 \times g$ for 10 min. More than 90% of the platelets were removed from the plasma by a further centrifugation at $400 \times g$ for about 30 min. Mononuclear leukocytes were collected from the interphase zone of an Isopaque-Ficoll gradient (Böjum 1968) after centrifugation of the cell fraction for 30 min at $400 \times g$.

A typical volume distribution of the leukocytes is shown in Fig. 4. The spectrum is measured with a Coulter Counter multichannel analyzer system (Coulter Electronics LTD).

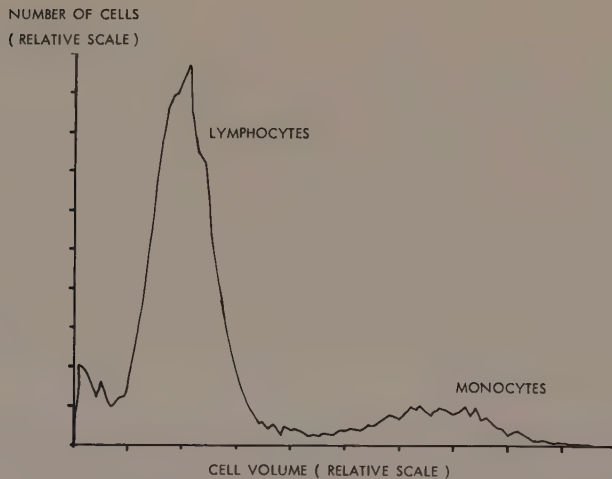


Fig. 4. Spectrum of human mononuclear leukocytes as obtained by the use of Isopaque-Ficoll separation.

The fraction of monocytes is normally around 20%. Cell cultures were

usually arranged to contain about 10×10^6 cells in 5 ml medium. The standard culture medium in most experiments was 4 ml Eagle's MEM with Hank's salts (Gibco) plus 1 ml autologous platelet poor plasma, pH 7.2-7.6.

(2) The use of UDS as an indicator of ADPRT activity (papers I and II). In paper I is described experiments on mononuclear leukocytes exposed to N-acetoxy-2-acetylaminofluorene (NA-AAF), ethylene oxide (EO), UV- and γ -radiation.

The NA-AAF was supplied gratis from Frederick Cancer Research Center (National Cancer Institute, USA) and dissolved in dimethyl sulfoxide (DMSO). Pure EO solutions (Merck) were diluted in Ringer's solution immediately before addition to the cell culture. The cells were exposed for these carcinogens at various concentrations for 1 h. The volume of the carcinogen solution was always smaller than $100 \mu\text{l}$ and the DMSO concentration in the NA-AAF experiment never exceeded 2%. After exposure the cells were spun down, washed and re-suspended in fresh medium.

For UV-irradiation the cells were suspended in 1 ml Ringer's solution and placed in a Petri dish with a diameter of 5 cm. The cell suspension layer was then about 0.5 mm thick and irradiated to various doses at a rate of $1 \text{ J} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ (R-52, Ultraviolet Products Inc.). Immediately after irradiation the cells were transferred to Falcon tubes and culture medium added.

γ -irradiation was performed at room temperature in a ^{137}Cs γ -ray source (Scandiatronix "Gammard 900", 0.8 Gy/min). The irradiation field was homogenous to 95% within a circle of radius 8 cm in horizontal plane and to ± 3 cm in vertical plane.

For estimation of DNA repair synthesis the culture media were supplemented with 10 mM hydroxyurea (HU) and tritium-labeled thymidine and the cells were incubated for 18 hr at 37°C in a carefully adjusted incubator.

HU at a concentration of 10 mM is known to suppress the ordinary repli-

cative DNA synthesis, but UDS (repair synthesis) is not significantly affected. In control experiments (paper I) it was shown that, as expected, this high concentration of HU did not influence the UDS induced by the genotoxic agents studied.

After appropriate repair incubation the cells were harvested by centrifugation. The DNA was extracted by a modified method of Marmur (Pero et al 1979). Briefly, the membranes were opened by sodium dodecyl sulphate, the nucleic acids extracted into the aqueous phase after mixture with CHCl_3 -isoamyl alcohol. The nucleic acids were adsorbed onto hydroxyapatite and then the ribonucleic acid preferentially removed by washing with 0.2 M phosphate buffer. The DNA was then removed in 0.4 M phosphate buffer, precipitated with 7% TCA and collected on nitrocellulose filters. The dried filters were counted in a toluene scintillation mixture. After counting the amount of DNA was determined by a diphenylamine procedure. The UDS was obtained as cpm $[\text{}^3\text{H}]\text{-dThd}/\mu\text{g DNA}$ after subtracting the background level of scheduled DNA synthesis determined in an untreated control culture.

(3) Measurement of UDS following hyperthermia and γ -radiation (paper II).

For these experiments, the cells were preincubated in water baths at various temperatures with controls at 37°C and then put on ice for irradiation. Incubation in an ice bath retards the DNA repair processes and makes it possible to compare results obtained, for example, with different dose rates.

After treatment, HU and $^3\text{H-dThd}$ were added, the cells incubated and UDS determined as above.

(4) Measurement of ADPRT activity (papers I and III). The cells were cultured in 4 ml Ringer's solution plus 1 ml autologous platelet poor plasma.

After treatment with the genotoxic agents and eventual repair incubation at 37°C , the cells were harvested. The cells were permeabilized in hypotonic

buffer. [^3H]-NAD $^+$, labeled in the adenine moiety and reaction buffer added using the procedure of Berger (1978c) with a few modifications as described in paper I. Following an incubation period of 15 min at 30°C the proteins were precipitated with ice cold TCA. The precipitates (containing ^3H -adenine ADP-ribosylated proteins - see Fig. 3) were then prepared for scintillation counting. Data were recorded as cpm [^3H]-NAD $^+$ /μg DNA.

(5) Measurements of nucleotide pools (papers II and III). Nucleotides were extracted and homogenized with perchloric acid and methanol as described in paper II. The neutralized supernatant was analysed by isotachopheresis on an LKB 2127 Tachophor equipment with an UV-absorbance detector. The principle of isotachopheresis is based on migration of ions in an electric field (see e.g. Everaerts et al 1976). The sample (ion mobilities of constituents m_{S_i}) is injected between a leading medium (ion mobility m_L) and a terminating medium (ion mobility m_T), so that the relation of the ion mobilities is given by $m_T < m_{S_i} < m_L$. The sample ions S_i ($S_1, S_2, \dots$) will then be separated according to their effective ion mobilities, which in turn are determined by shape and charge of the ion, solvation, dielectric constant and viscosity of the solvent, pH, degree of dissociation and temperature. Utilization of intermediate mobile compounds with effective ion mobilities between those of the ion species to be separated (i.e. so called spacers) result in UV-absorbance spectra with discrete peaks whose areas correspond to the quantity of nucleotides. After calibration, nucleotide concentrations were obtained in pmol/10 6 cells.

(6) Estimation of cell viabilities (paper II). A convenient criterium of "cell viability" is the ability of the cell to exclude certain dyes such as trypan blue "the trypan blue exclusion method". However, as pointed out by Schrek (1965) the number of cells capable of division (i.e. "vitality" of the cell)

is probably less than the number of viable cells measured by a dye exclusion test. Measurement of the reproductive death involves methods of bioassay techniques, i.e. cloning capacity. However, cytotoxic effects of hyperthermia on various normal and malignant cell types measured by dye exclusion test have also been confirmed by bioassay methods (Schrek 1979).

We have used the trypan blue exclusion method since mononuclear leukocytes are mainly in the G0 phase of the cell cycle. The PHA stimulation method is usually not suitable for this type of experiments since this means addition of mitogenic substances which in itself may interfere with the treatments given.

We have found (Fig. 7 in paper II) that damage to mononuclear leukocytes is not immediately apparent on trypan blue dye exclusion. Damage to the cells is first seen after about 10 h incubation at 37°C. After 24 h incubation at 37°C the effect on viability of the various treatments can be evaluated with good reproducibility.

B. IN VIVO experiments using a transplanted C3H'mouse mammary adenocarcinoma (papers V and VI).

The background of these experiments was to test the radiosensitizing potential of one inhibitor of ADPRT in vivo.

The model system used was C3H mice with transplants from a single mouse mammary adenocarcinoma occurring spontaneously in this type of mouse. The tumor had successively been transplanted for 30 generations before these experiments. The tumor line was kindly supplied to us from Department of Tumor Biology, Karolinska Institutet in Stockholm.

Viable pieces were selected and pressed through a Borell strainer into Parker tissue medium (Gibco). After washing in medium the cell suspension was adjusted to about 2×10^6 cells/ml and 0.15 ml aliquot injected subcutaneously above the right gastrocnemius muscle of full-grown C3H mice who were about 2 months old and had average body weights of about 20 g. Tumor take was nearly 100%. The tumors were usually palpable after 5 days. The tumors were regarded as prolate spheroids with minor and major axis a and b , respectively, measured in the sagittal plane of the animal. The tumor volume (V) is then given by

$$V = \frac{a^2 b}{2}$$

In our papers we simply compared average growth as given by the tumor volumes of the untreated controls, to those given various doses of radiation and to those given ADPRT inhibitor (i.e. nicotinamide) plus various doses of radiation at various times after treatment.

Histologic studies of tumors after various treatments are reported in paper VI.

Effects of the various treatments were also studied on normal tissue in a so called tail stunting experiment. The stunting of growth of tail vertebrae of baby mice (or rats) is a convenient parameter for assessing

the effects of radiation and sensitizers on normal tissue (Wright and Howard-Flanders 1956, Dixon 1967, Field et al 1977, Hahn et al 1980, Hume et al 1982). Seven days old BALB mice were given the same dose of ADPRT inhibitor (i.e. nicotinamide, 0.2 g/kg), intraperitoneally and the tails were irradiated. The length of the tails were then followed for about 2 months and then the various treated sub-groups were compared.

Nicotinamide was chosen as an inhibitor of ADPRT because it is essentially free from side effects and very high doses can be tolerated by the experimental animal. Nicotinamide at a concentration of 2 mM has been found to be optimum to inhibit ADPRT in cultured cells treated with genotoxic agent (Berger and Sikorski 1980a). This concentration corresponds to about 0.2 g nicotinamide per kg, which is far below the $LD_{50}=1.68$ g/kg found for rats (MERCK INDEX, 9 ed 1976). This dose was not lethal for C3H mice. The nicotinamide was injected intraperitoneally about 45 min before irradiation in 200 μ l aliquots as a 0.2 M saline solution. This time was shown in paper VI to be optimum for distribution of nicotinamide within the animals.

7. RESULTS AND REVIEW OF THE PAPERS

Paper I. Here are compared, in resting human mononuclear leukocytes, the effect on DNA repair, as measured by UDS, and on ADPRT activity following DNA damage by γ -radiation, UV-radiation, EO and NA-AAF. Inhibition of ADPRT by nicotinamide and benzamide result in considerable increase of UDS induced by γ -radiation, UV-radiation and EO. However, the level of NA-AAF-induced UDS was not affected by these ADPRT-inhibitors.

Many authors have found that ADPRT inhibitors stimulate UDS (Berger and Sikorski 1980, Althaus et al 1980, Miwa et al 1981, Durkacz et al 1981). However, Jacobson et al (1983) argue that the stimulatory effect of ADPRT inhibitors observed might be due to the presence of the high concentration of HU (10 mM) used to suppress the background of replicative DNA synthesis. Jacobson et al found namely that 10 mM HU alone reduced ^3H -thymidine incorporation in normal human diploid fibroblast CF-3 cells irradiated with 5 J/m^2 UV by 36%. However, in the presence of 3 mM 3-aminobenzamide the DNA repair replication capacity was not inhibited by HU. Thus, only in the presence of HU, 3-aminobenzamide caused an apparent stimulation of repair replication.

We note in our work at this low dose of UV radiation (5 J/m^2) only a small stimulation of UDS in the presence of 2 mM nicotinamide (about 10% increase). The stimulation of UV-induced UDS increased with increasing dose of UV radiation (about 60% stimulation at 20 J/m^2). We find at this high UV dose a similar stimulating effect on UDS by 2 mM nicotinamide in the presence of HU at a concentration as low as 1 mM. This was also true for the other DNA damaging agents studied, γ -radiation, EO and NA-AAF, where we found almost the same UDS levels in the presence of 1 mM or 10 mM HU. Thus our data indicate that at least at high doses of DNA damaging agents, the concentration of HU does not significantly affect the UDS level obtained in the presence or absence of ADPRT inhibitors. Similarly Sims et al (1982) found that 10 mM

HU did not alter nicotinamide-induced (2 mM) stimulation of UDS in lymphocytes damaged by 50 J/m^2 UV-radiation or $136 \mu\text{M}$ N-methyl-N'-nitro-N-nitrosoguanidine.

The ADPRT activity was stimulated in a dose dependent manner with γ -radiation, UV-radiation and EO but NA-AAF did not induce any significant enzyme activity.

Our hypothesis is that the absence of NA-AAF stimulation of UDS may be explained from the number of DNA strand breaks that exist at any time during excision repair is low because the rate limiting event in excision repair is recognition of damage. NA-AAF damage to DNA is badly recognized and endonuclease activity will then be low. Hence, inhibition of ADPRT has only a small effect on NA-AAF-induced UDS because of the few DNA strand breaks that accumulate, and as a result low activation of the enzyme occurs.

Paper II. Effects of γ -radiation and hyperthermia on DNA repair synthesis (UDS) and on the levels of nucleotide pools in cultured human mononuclear leukocytes were investigated.

It was found that γ -radiation induced UDS decreased with increasing temperature (37-45°C). The reduced repair capacity found following hyperthermia treatment could be due to reduced DNA glycosylase or exonuclease activity, reduced intracellular levels of nucleotide pools or due to less effective polymerase β activity. Spiro et al (1982) have studied the effect of hyperthermia on polymerase α and polymerase β activities in CHO cells. They found a correlation between inhibition of UDS and loss of polymerase β activity. Inactivation of polymerase β could be due to thermal denaturation of the enzyme or from changes in the intracellular milieu resulting from damage of other cellular structures (e.g. biomembranes, ionic levels, etc) (Spiro et al 1982, 1983).

The nucleotides were measured with the aid of isotachophoresis. One important advantage of this method is that many nucleotides can be measured in one experiment. However, many cells ($10\text{-}20 \cdot 10^6$) are needed to get data with good accuracy. Our data are compared with corresponding values published in the literature (Table I):

Table I.

CONCENTRATION OF RIBONUCLEOTIDES IN RESTING HUMAN MONONUCLEAR LEUKOCYTES (LYMPHOCYTES).

Values in pmole/ 10^6 cells ($\pm$ S.D.). Number of individuals used within brackets.

Nucleotide	Paper II of this thesis [18]	Berger et al (1982b) [20]	deAbreu et al (1982) [21]	Peters et al (1983) [11]
AMP	69 ± 11	-	25 ± 11	43 ± 26
ADP	211 ± 46	-	53 ± 40	302 ± 64
ATP	1140 ± 188	-	500 ± 140	855 ± 159
Energy charge*	0.88 ± 0.02	-	0.90 ± 0.04	0.84 ± 0.02
ATP/ADP	5.4 ± 0.5	-	13 ± 7	2.9 ± 0.5
NAD ⁺	118 ± 17	69.9 ± 21.7	-	102 ± 89

* Energy charge = $\frac{1}{2} \frac{[ADP] + 2[ATP]}{[AMP] + [ADP] + [ATP]}$ (Atkinson 1968).

All authors used blood collected in heparin except Berger et al (1982b), who defibrinated the blood and removed the platelets by swirling with glass beads. Peters et al (1983) and deAbreu (1982) used high performance liquid chromatography for quantitating the nucleotides. Berger et al (1982b) measured the pyridine nucleotides by enzymatic cycling techniques. The different nucleotide levels obtained might be due to the different techniques used and contaminations of the leukocytes with platelets or a variable fraction of monocytes present. This point indicates the absolute necessity of preparing the cell cultures as similar as possible for a succession of experiments.

Following γ -ray damage the NAD^+ pools drop to very low values but return to their original level within about 5 hrs. After moderate hyperthermia (42.5°C for 45 min) and γ -ray damage, the NAD^+ pool recovered only to about 70% of the original level even after 8 hrs repair incubation at 37°C . Following treatment with 44°C prior to γ -radiation the cells did not recover at all. It was also shown that ATP pools following 30 Gy γ -radiation were depleted but regenerated at normal temperatures (i.e. 37°C). When hyperthermia was given prior to radiation, the cells were unable to restore their pools; the higher the temperature the larger the lack of regeneration.

Hyperthermia in combination with γ -radiation also resulted in reduced viability of the leukocytes compared to these treatments alone. The data clearly demonstrate the correlation between DNA repair and poly ADP ribosylation. When the demand for NAD^+ is great because of excessive DNA strand breaks following γ -irradiation, then the function of ADPRT becomes critical. A limited supply of ATP during peak ADPRT activity limits the regeneration of NAD^+ , which in turn means less substrate for the enzyme and increased cytotoxicity from the radiation (see page 21). It should also be noted that NAD^+ is a co-factor in numerous cellular reactions. Maintaining the level of NAD^+ is therefore of greatest importance for cellular life.

Paper III. Our hypothesis from paper II regarding one possible effect of hyperthermia on DNA damage and repair is further investigated here. The report describes effects on ADPRT activity, NAD^+ and ATP levels and ultra-structure in human mononuclear leukocytes subject to irradiation and heat. γ -radiation was found to activate ADPRT considerably, but we find no further effect on the activation level when the cells were exposed to hyperthermia prior to radiation. Our results on leukocytes are in agreement with data obtained by Juarez-Salinas et al (1984), who measured ADPRT activity in SVT2 cells following hyperthermia treatments as high as 45°C . These authors found no significant changes in enzyme activity although there were large accumulations of poly (ADP-ribose). However, Noland and Kidwell (1983) found for Drosophila nuclei where the temperature optimum is 20°C that ADPRT activity was quite lost at 37°C . Similarly, Kidwell and Purnell (1983) reported that exposure of HeLa cells to 43°C resulted in inactivation of ADPRT.

Juarez-Salinas et al (1984) argue that hyperthermia by itself does induce some DNA fragmentation which could activate the enzyme enough to yield the poly (ADP-ribose) measured. The authors further suggest that hyperthermia might inactivate the (ADP-ribose)-polymer-degrading enzyme poly (ADP-ribose) glycohydrolase (see page 21) which then would also result in a high level of poly (ADP-ribose). In order to test the role of ATP as one precursor to NAD^+ and its role on poly (ADP-ribose) metabolism, we uncoupled the oxidative phosphorylation with 2,4-dinitrophenol (DNP) and examined the level of NAD^+ after damaging the cells with γ -radiation. Interestingly, we find that cells given γ -radiation did not get reduced NAD^+ pools unless glucose was omitted from the culture medium. Our data indicate that ATP production from glycolysis is sufficient to prevent NAD^+ depletion from γ -radiation but not after hyperthermia treatment. We also show that exogenously supplied adenosine could not be fully utilized for ATP production after combined treatment of the leukocytes with hyperthermia and γ -radiation.

The large decline of NAD^+ observed following treatment with heat and γ -radiation is important because the level of NAD^+ then probably will be rate limiting for ADPRT activity. The NAD^+ pool in the mononuclear leukocytes is about $120 \text{ pmole}/10^6 \text{ cells}$ (Table I page 40). With a volume of the leukocytes of about 300 fl , we find the NAD^+ concentration to be about $400 \text{ }\mu\text{M}$. At least at the reduced levels we obtain after damaging the cells with hyperthermia and radiation, ADPRT-activity will not be saturated, the K_m value in permeabilized cells being $328 \pm 127 \text{ }\mu\text{M}$ (Durkacz et al 1980). It should be mentioned that NAD^+ pools and ATP metabolism are coupled via equilibrium relations so that the red-ox NAD^+/NADH -ratio fits its function in the energy producing systems. Hence, ATP production is also limited and this limitation may also play a role in other steps involving DNA repair (Kaufmann et al 1982, Dresler and Lieberman 1983).

When looking at the morphology of lymphocytes with electron microscope, it was found that mitochondrial cristae and endoplasmic membranes are dilated following combined treatments with heat and γ -radiation. This point further supports the hypothesis that hyperthermia primarily affects energy production in mononuclear leukocytes. As one mechanism of the action of hyperthermia, we suggest that hyperthermia induces a sequence of events whereby suppression of ATP production leads to NAD^+ decline and substrate starvation for ADPRT. Thus it is possible that hyperthermia and ADPRT inhibitors act in a similar way to influence DNA damage and/or repair. Inhibitors of the enzyme (i.e. by nicotinamide) or suppression of substrate regeneration for the enzyme (i.e. by hyperthermia) might mean accumulation of γ -ray-induced DNA damage and increased cytotoxicity.

Heat has been found to reduce cellular ATP also in other cell lines (Francesconi and Mager 1979, Ohyama and Yamada 1980, Lunec and Cresswall 1983). Findly et al (1983) report 50% decrease in ATP levels of Tetrahyma species exposed to heat which correlated well with simultaneous synthesis of

heat shock proteins (page 15). From their data the authors conclude that the main effect of hyperthermia is hydrolysis of ATP to free nucleosides. Ohyama and Yamada (1980) argue that heat might both stimulate ATP synthesis and cause its degradation:

However, decrease of ATP is probably not a universal response of cells following heat treatment. Henle et al (1984) found no significant depletion of ATP in HeLa cells given hyperthermia, although there was a strong decrease of survival at high temperatures (Henle and Dethlefsen 1978, Henle et al 1984). Also in Ehlich ascites cells energy production appears to be more heat resistant than survival. Gerweck et al (1984) found no immediate decrease in ATP following treatment with moderate hyperthermia. This obvious differential sensitivity of various cell types to heat may be of importance when identifying target cells for hyperthermia effects.

Paper IV. Numerous studies support the hypothesis that the plasma membrane is one critical target for heat inactivation of cells (see page 14). Not only is the membrane lipid composition affected but also the morphological structure is changed. This means that diffusion, permeability and other transport processes across the membranes can be disturbed.

In this paper levels of some intracellular ions are measured after exposure of human lymphocytes to hyperthermia up to 46°C . At temperatures above 43°C the level of K decreased rapidly with increasing temperature and there was a simultaneous but smaller gain of Na. Unfortunately, the level of Cl could not be measured. We find no significant effect of heat on protein bound ions.

The heat ion currents across the cell membrane are, of course, related to each other to conserve electrical charge. Our results, together with data by Yi et al (1983a), indicate that, following heat treatment (above 42.5°C), there must be an inflow of hydrogen ions into the cell which will lower the intracellular pH. Several studies show that hyperthermia strongly influences the intracellular pH with a shift to lower pH the higher the temperature (see e.g. Gerweck 1977). Since pH in tumors is about 0.5 units lower than in normal tissue (Bicher et al 1980), a further reduction of tumor pH might lead to increased lethality.

In line with earlier papers discussed above, we argue that the reduced efficiency of ion pumping observed might be due to suppressed ATP production in the lymphocytes, although other mechanisms must also be considered. One possible mechanism for the observed effects could be diffusion of the ions along their concentration gradients. A third explanation was suggested by Yi et al (1983b). They found that the total intracellular pool of amino acids increased significantly after hyperthermia treatment of mastocytoma cells. The increased pools of amino acids could then act as a source of osmolarity maintaining the cellular volume almost intact. The efflux of K following

hyperthermia was then interpreted as a cellular response to the increased osmolarity.

This paper is also methodological and describes the method of Particle-Induced X-ray Emission (PIXE). Ionic levels in lymphocytes measured by the PIXE method are compared with similar data obtained from the same blood donors with atomic absorption spectrometer. We stress in the paper the role of this very sensitive PIXE method as a tool for routine multi-elemental trace analysis for biological samples.

Lymphocytes are very suitable cells to study because they are easy to collect and they are quite metabolically active. It is possible that lymphocytes, rather than plasma or serum concentrations of ions, reflect much better the intracellular or total body levels of elements. For example, lymphocyte K levels have also been found to reflect the K status of cardiac and skeletal muscle (Counihan et al 1978). In addition, patients with chronic congestive heart failure treated with loop block diuretics have been reported to have significantly reduced lymphocyte Mg and K levels compared to control subjects (Ryan et al 1978).

Papers V and VI. The apparent role of ADPRT inhibitors as potentiators of lethality of DNA damaging agents suggest experiments investigating eventual effects of nicotinamide as a radiosensitizer also in vivo.

Already before the role of ADPRT was known one experiment was carried out by Calcutt et al (1967) where nicotinamide was found to function as a radiosensitizer on sarcoma S.180 tumors transplanted to BALB/C mice. Of 25 animals pretreated with nicotinamide (400 mg/kg given intraperitoneally 3.5 hrs before irradiation with 45 Gy), 24 regressed completely leaving only a scar. Also the $LD_{50/30}$ -dose was lowered with about 0.5 Gy when the animals were irradiated 3.5 hrs after treatment with nicotinamide. For control animals not given nicotinamide the $LD_{50/30}$ -dose was found to be 7.15 Gy. Calcutt et al (1967) further found that nicotinamide at 450 mg/kg caused a slight increase in weight of the mice compared to untreated controls, indicating lack of direct toxic effects of nicotinamide.

In our experiments we used transplanted C3H mouse mammary adenocarcinomas as a model system. Five days after transplantation the tumors were usually easily palpable.

There is a wide variation in tumor growth from animal to animal. Fig. 5 shows the average tumor volumes versus time after transplantation for the untreated control animals from papers V - Group 1 and VI - Group 2.

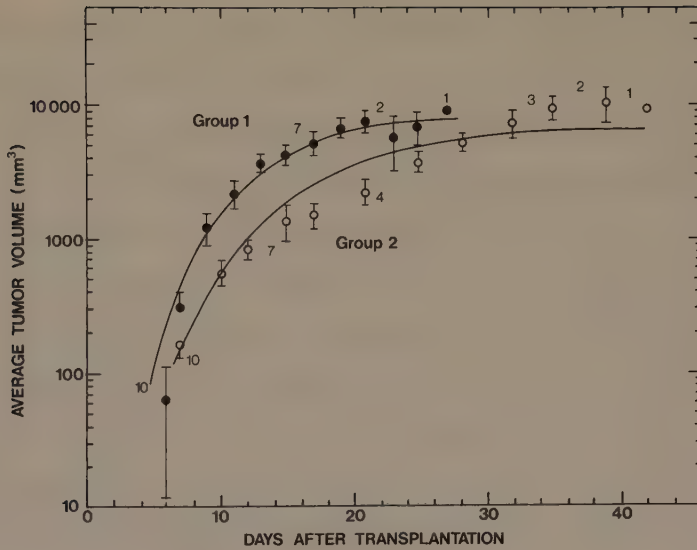


Fig. 5. Average tumor volume versus time after transplantation of untreated control animals. Group 1 from paper (V) and Group 2 from paper (VI). Error bars indicate $\pm$ SEM. The numbers next to data points show how many mice were living at that time.

Characteristically the growth rates of the untreated tumors are at first rapid, almost exponential, and then slow off progressively. The curves in Fig. 5 are least squares fits to the experimental data of a Gompertz formula* (Laird 1964):

$$V = V_0 \exp \frac{A}{\alpha} [1 - \exp (-\alpha t)] ,$$

where V is the tumor volume (mm^3) at time (hrs) after transplantation. V_0 is the extrapolated "tumor volume" at time zero. A and α are constants; A related to the initial growth and α a retarding factor. This formula has been found

*Gompertz (1825) originally deduced this type of formula from studies of human mortality and age.

to very well describe the growth pattern of many animal tumors (Laird 1964, 1965, McCredie et al 1965). At initial growth $[1 - \exp(-\alpha t)] \approx \alpha t$, which gives the formula for exponential growth $V = V_0 \exp(A \cdot t)$. The three parameters from the two set of data are given in Table II.

Table II

C3H tumor	A (hrs ⁻¹)	α (hrs ⁻¹)	V_0 (mm ³)
Group 1	0.10 ± 0.06	0.008 ± 0.002	0.04
Group 2	0.06 ± 0.04	0.006 ± 0.002	0.3

These parameters can be compared to similar data reported by Laird (1964, 1965) and McCredie et al (1965). With a cell diameter of 10 μm , the volume of tumor cells transplanted is about 0.2 mm³, which corresponds roughly to the V_0 -values in the table!

The tumor doubling time T_{x2} at time t after transplantation is given by the parameters in the Gompertz formula as

$$T_{x2} = -\frac{1}{\alpha} \ln \left[1 - \frac{\alpha \ln 2}{A} \exp(\alpha \cdot t) \right].$$

Doubling times for our two experimental groups are shown in Fig. 6 versus age of tumor.

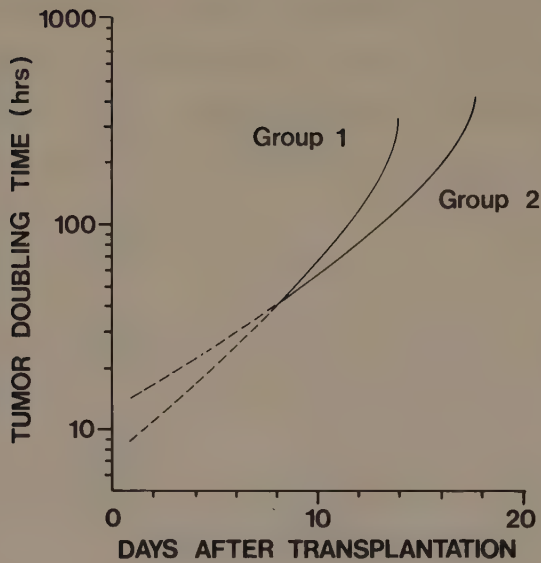


Fig. 6. Tumor doubling time versus age of tumor for the two animal groups studied.

The reason for increasing doubling time with age of tumor could be due to: (i) increasing duration of cell cycle time with tumor growth, (ii) decreasing fraction of proliferating cells, (iii) increasing loss of cells from tumor volume (cell death and dead cell resorption) and (iv) combinations of (i) - (iii).

When analysing the percentage of labeled mitoses in a population at various times after injection of radioactive label incorporated in DNA, one usually find no discrete cell cycle time but a cell cycle time distribution among the proliferating cells. Frindel et al (1967) concluded from their and other data that the average duration of cell cycle is probably a characteristic of the type of cell which does not change during tumor growth. Denekamp (1970) reported for various carcinomas that there is no significant variation of the cell cycle times with their doubling times. For C3H mouse mammary carcinoma Denekamp (1970) found that the average duration of cell cycle is approximately 100 hours, which is in good agreement with the doubling times of the tumor.

found an average cell cycle time of about 15 hrs with a large spread. If we use the Gompertz formula for small t , i.e. where we have exponential growth, a rough estimate of cell cycle time (T_C) is expected to be given by $T_C = \frac{\ln 2}{A}$, which for our experimental animal groups 1 and 2 give $T_C \approx 7$ and 12 hrs respectively, which values seem too short to be realistic (Bresciani 1965). Anyway, cell cycle time appears very short compared to late tumor volume doubling times, which means that other factors must be of importance for growth.

The growth fraction, defined as the ratio of proliferating cells (i.e. actively involved in the mitotic cycle) to the total number of proliferating and non-proliferating cells in the population, seems fairly constant with tumor volume doubling times, being about 50% for a big material of sarcomas and carcinomas (Denekamp 1970). Mendelsohn (1962) report for multiple auto-transplants of C3H mammary tumors a growth fraction of about 40%. About the same value was found for undisturbed primary tumors with no correlation between growth fraction and tumor size. Denekamp (1970) found for C3H a growth fraction estimated with two different methods to be 30 and 37% respectively.

Thus it is to be expected that the fraction of cells which are lost from tumor volume should increase with tumor doubling time. This has also been found for various sarcomas and carcinomas (Denekamp 1970).

Of importance for the factors determining tumor growth is the vascular capacity for supply of oxygen and nutrients to the tumor cells. A necrotic center will develop as the tumor outgrows from its blood supply (see Fig. 1 in paper VI). Tumor cells will grow only in a thin layer around the necrosis. Immunological factors might also be of importance for tumor growth and cell loss.

When irradiated with a single dose the tumors shrink at least temporarily followed by regrowth (Figs. 1 and 2 in papers V and VI respectively) (Suit and

Walker 1980). When nicotinamide was administered prior to radiation there is for high doses of radiation even more prominent shrinkage and a clear delay of regrowth. Other endpoints studied are tumor volume ratios and increase of life span. In paper VI we also study the effect of the combined treatment on normal tissue in a tail stunting experiment utilizing BALB/CA mice.

One widely used endpoint in studies of radiosensitizing agents is the TCD_{50} value (Begg et al 1974, Sheldon et al 1974, Fowler et al 1974). The TCD_{50} dose is the dose at which 50% of the tumors are locally controlled (e.g. cure of patient or animal) which then could be used to give radiosensitizing enhancement ratios. To get accurate TCD_{50} values, the curative effects must be studied at many doses of radiation and followed for long time periods after treatment. However, a rough estimate of TCD_{50} values was deduced from the tumor remission data valid one month after treatment (Table I in paper VI). Using Poisson distributions for tumor remission probability (Overgaard 1985, personal communication) we find TCD_{50} values being about 36 Gy for γ -radiation alone and 29 Gy for the combined treatment of nicotinamide plus γ -radiation. These values give a nicotinamide-induced enhancement ratio of about 1.2, which is comparable to radiosensitization by metronidazole in the same animal model system (Begg et al 1974).

The collected data of papers V and VI indicate a radiosensitizing effect of nicotinamide on tumors as well as on normal tissue.

Normal tissue damage is the major limiting factor in any approach to treatment of malignant tumors. However, it is reasonable to believe that ADPRT inhibitors should have larger effect on tumor tissue because of the lower level of NAD^+ found in a variety of tumors, fetal tissue and regenerating liver compared to normal and adult tissue (Jacobson and Jacobson 1976) (see also page 22).

Thus our results indicate a possible use of ADPRT inhibitors together with γ -radiation (or cytotoxic drugs) in the treatment of malignant tumors.

8. SUMMARY AND CONCLUSION

The molecular mechanisms behind the effect of hyperthermia and ADPRT inhibitors interfering with DNA damage and/or repair have been studied. Human mononuclear leukocytes in vitro and a C3H mouse mammary adenocarcinoma in vivo were used as model systems. The following parameters were studied in vitro:

- (1) DNA repair capacity was measured using the assay system of quantifying UDS; i.e. measuring incorporation of ^3H -thymidine into DNA of non-S phase cells.
- (2) ADPRT activity with and without the presence of inhibitors of the enzyme was studied following DNA damage induced by various agents.
- (3) The intracellular level of NAD^+ , which is a co-substrate for ADPRT during DNA repair, was measured together with the pools of ATP.
- (4) Membrane effects of hyperthermia were studied by measuring intracellular ionic levels (especially potassium).
- (5) Cell viability was estimated with trypan blue exclusion method.

The data indicate that these parameters are sensitive to heat, at least, above 43°C where there is a strong decrease in the level of each parameter with increasing temperature. Obviously there exists an onset of some fundamental inactivation mechanism at this temperature, which we have suggested limits ADPRT activity.

We argue that hyperthermia and ADPRT inhibitors might act via the same mechanism in preventing poly ADP-ribosylation of one or more chromosomal proteins. ADPRT inhibitors directly prevent poly ADP-ribosylation whereas hyperthermia mainly affects ATP production (at least in the mononuclear leukocytes studied here) and as a result the regeneration of NAD^+ , which in turn means less substrate for poly ADP-ribosylation. Reduced pools of NAD^+ following

treatment with heat and radiation is important since the level of NAD^+ will then probably be rate limiting for ADPRT activity.

The mechanism of rapid (re)generation of NAD^+ for ADP-ribosylation of chromosomal proteins might be of greatest importance in regulation of DNA damage and/or repair.

As a model for this hypothesis, we have demonstrated in vivo that C3H tumors are radiosensitized in the presence of the ADPRT inhibitor nicotinamide.

If there proves to be some additivity of the toxic effects of ADPRT inhibitors and hyperthermia when combined with γ -radiation (or cytotoxic drugs) then this would be of great importance for improving the results of the treatment of malignant tumors.

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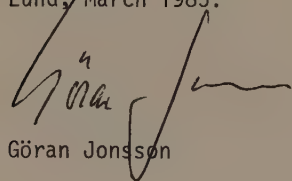
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Lund, March 1985.

A handwritten signature in black ink, appearing to read 'Göran Jonsson', with a long horizontal flourish extending to the right.

Göran Jonsson

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Papers I-VI

UNSCHEDULED DNA SYNTHESIS INDUCED BY *N*-ACETOXY-2-ACETYLAMINOFLUORENE IS NOT SENSITIVE TO REGULATION BY ADP-RIBOSYL TRANSFERASE

RONALD W. PERO^a, GÖRAN G. JONSSON^{a,b} and LENNART PERSSON^a

^a*Biochemical and Genetic Ecotoxicology, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund and* ^b*Department of Oncology, University Hospital, S-221 85 Lund (Sweden)*

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SUMMARY

We have directly compared in resting human mononuclear leukocytes the DNA repair effects caused by ADP-ribosyl transferase (ADPRT) activity following DNA damage induction by gamma radiation, UV radiation, ethylene oxide (EO) and *N*-acetoxy-2-acetylaminofluorene (NA-AAF). The presence of inhibitors of ADPRT during the quantitation of unscheduled DNA synthesis (UDS) resulted in about a 2-fold increase of UDS when induced by gamma radiation, UV radiation or EO. The stimulation of UDS by EO, UV- or γ -radiation in the presence of an ADPRT inhibitor was equally strong whether 1 mM or 10 mM hydroxyurea was used to suppress scheduled DNA synthesis. The level of NA-AAF induced UDS was not affected by inhibitors of ADPRT. In addition, direct estimation of ADPRT activity revealed that at doses giving maximal UDS, NA-AAF damage did not induce a measurable enzymatic activity whereas γ -radiation, UV radiation and EO all showed a significant dose response increase. We have interpreted our data to mean that NA-AAF induced UDS estimates DNA repair relating mainly to DNA lesions that are recognized with difficulty, and hence, the rate of endonuclease-induced DNA strand break accumulation is not sufficient to allow a stimulation of ADPRT and affect the quantitation of UDS.

Key words: Excision DNA repair — Enzymatic DNA repair — Human lymphocytes — DNA damage

Abbreviations: ADPRT, adenosine diphosphate ribosyl transferase; EO, ethylene oxide; NA-AAF, *N*-acetoxy-2-acetylaminofluorene; TCA, trichloroacetic acid; UDS, unscheduled DNA synthesis.

INTRODUCTION

ADPRT is a chromatin-associated enzyme that catalyzes the formation of the homopolymer poly(ADP-ribose) attached to nuclear proteins, principally histones [1]. The coenzyme NAD^+ is an absolute requirement for ADPRT activity since the ADP-ribose moiety of NAD^+ is used in the reaction to form poly(ADP-ribose). The function of the enzyme is unknown but the evidence is rapidly accumulating in favor of some role in the process of DNA repair.

So far it has been shown that ADPRT activity is stimulated by induced DNA damage [2–5] and could be correlated to: (i) repair of DNA strand breaks [3,6], (ii) cytotoxicity from genotoxic exposures [3–5], (iii) the level of sister chromatid exchanges [7], (iv) the level of UDS [5,8–11], (v) the control of endonuclease [12–14] and ligase [15] activities, (vi) the repair capacity of individuals with xeroderma pigmentosum [16,17] and Fanconis Anemia [18] and (vii) cellular differentiation [19,20] and gene expression [21].

Recently, Cohen and Berger [22] have determined that chromosomal DNA damage per se (i.e. damage which does not directly result in DNA strand breaks such as UV-radiation) does not stimulate ADPRT activity. However, if simian virus 40 minichromosomes were UV-irradiated and then nicked by addition of UV endonuclease, the enzyme was stimulated to a higher level of activity. These results suggested to us that DNA damaging agents which require excision endonuclease activity for repair may influence ADPRT activity quite differently, if there are substantial differences in the kinetics of DNA repair of these various types of DNA lesions.

Our laboratory has been concerned with the quantitative assessment of UDS in human mononuclear leukocytes. We have searched for interindividual differences in UDS following a standardized dose of NA-AAF. Our data have indicated that NA-AAF induced UDS varies in accordance with age [23–25], sex [24], blood pressure [23–25], general health status [25], genetic predisposition for colon cancer [26] and occupational exposure to genotoxic agents [27–29]. However, when we used UV induced UDS to assess interindividual variation, the method was difficult to quantitate and consistent correlations were difficult to achieve by us [30] and others [31,32].

One reason we offered for this discrepancy was the considerable differences in DNA repair kinetics that existed following stimulation of UDS from UV- and NA-AAF-induced-DNA damage [30]. The UV induced UDS was 50% completed in 2.5 h whereas it took 7 h to complete 50% of NA-AAF-induced UDS. Therefore, as ADPRT has been shown to alter UDS, and as the enzyme required DNA strand breaks and not just damaged DNA, then we decided to examine if DNA damaging agents, which induce quite different rates of excision repair synthesis, would differ in their capacities to stimulate ADPRT. Here we report that the quantitation of NA-AAF induced UDS is not affected by inhibitors of ADPRT nor did NA-AAF

damage in resting human mononuclear leukocytes induce a detectable activity of this enzyme.

MATERIALS AND METHODS

Isolation of lymphocytes

Heparinized vacutainers (143 U.S.P. units/tube) were used to take peripheral blood samples by venal puncture. The heparinized blood was centrifuged at $100 \times g$ for 10 min and the platelet rich plasma removed. The platelets were then removed from the plasma by centrifuging at $400 \times g$ for 25–30 min. The platelet poor plasma was used as a culture supplement in these experiments. The blood cell pellet was diluted to its original volume with physiologic saline and then the sample was layered onto an Isopaque-Ficoll cushion and spun at $400 \times g$ for 25–30 min. The lymphocytes were isolated from the interphase zone of the gradient [33].

Measurement of UDS in the presence of ADPRT inhibitors

The methods for estimating UDS in resting human mononuclear leukocytes following exposures to NA-AAF [23], UV irradiation [30] and EO [27] have already been reported in detail. Briefly, the cells were given the various exposures reported in Fig. 1 but in the absence of either nicotinamide or benzamide, and then, they were harvested by centrifugation and resuspended in 20% autologous plasma supplemented Hank's Eagle medium. The cell cultures were then incubated for 18 h at 37°C in the presence and absence of either 2 mM nicotinamide or 2 mM benzamide, 10 $\mu\text{Ci/ml}$ [^3H]dThd (25 Ci/mmol) and 10 mM hydroxyurea. γ -Irradiation of lymphocytes was carried out at room temperature in 20% autologous plasma supplemented Hank's Eagle medium containing 10 $\mu\text{Ci/ml}$ [^3H]dThd, 10 mM hydroxyurea, and either in the presence or absence of 2 mM nicotinamide. Following γ -irradiation the cell cultures were incubated at 37°C for 18 h. The details of the biochemical determination of UDS was performed as we have described [23]. The data were recorded as cpm [^3H]dThd incorporated per μg DNA minus the [^3H]dThd incorporated from hydroxyurea-suppressed replication synthesis.

Measurement of ADPRT activity

Cell permeabilization and measurement of poly(ADP-ribose) synthesis were performed with only minor modifications according to Berger [34]. Approximately 10×10^6 mononuclear leukocytes per treatment were exposed to either 0–100 Gy γ -radiation, 0–300 J/m² UV radiation, 0–25 mM EO or 0–80 μM NA-AAF in 5 ml of 0.9% NaCl supplemented with 20% autologous platelet poor plasma. Cells were exposed to gamma radiation in an ice bath and UV exposure was completed within only a few minutes at room temperature. Irradiated cells were then incubated for an additional 90 min at 37°C in order to allow a comparison of the exposure times necessary when using chemical agents such as EO or NA-AAF. Hence, cells were

exposed to EO and NA-AAF by incubating the cell cultures continuously at the desired doses for 90 min at 37°C. Following the various genotoxic exposures, all samples were centrifuged at $300 \times g$ for 10 min, the cell pellets suspended in 1.5 ml of permeabilization buffer (0.01 M Tris-HCl, pH 7.8; 1 mM EDTA; 30 mM 2-mercaptoethanol; 4 mM MgCl_2), and left on ice for 15 min. After permeabilization the cells were collected by centrifugation at 4°C. The cells were resuspended in 50 μl of permeabilization buffer, 25 μl of poly(ADP-ribose) reaction buffer (100 mM Tris-HCl, pH 7.8; 120 mM MgCl_2), 15 μl of $[^3\text{H}]\text{NAD}^+$ (155 μM , 150 mCi/mmol, ^3H -label in adenine) and the mixture was incubated for 15 min at 30°C. The reaction was terminated by the addition of 0.5 ml of 3.7 M NaCl and 10 μl of 25% sodium dodecyl sulfate. The samples were heated for 30 min at -60°C to obtain a more homogeneous cell lysate. Two 230- μl aliquots of the lysate were each precipitated with 1 ml of cold 10% trichloroacetic acid (TCA) and the resultant suspensions were immobilized onto nitrocellulose filters (Millipore, HAWP 01300). The filters were washed with $3 \times 1 \text{ ml}$ 10% TCA, $2 \times 1 \text{ ml}$ ethanol solution (0.1 M sodium acetate in 66% ethanol), dried, the radioactivity counted, and the DNA quantified as previously described [23]. The data were recorded as cpm $[^3\text{H}]\text{NAD}^+$ incorporated into TCA precipitable material per μg DNA. We have viewed basing our data on the amount of DNA as a means of correcting for variations in cell number from sample to sample.

RESULTS

We have selected 4 DNA damaging agents to assess the role of ADPRT activity in relation to UDS. UV radiation and NA-AAF induce large patch repair (about 100 nucleotides) and both require endonuclease activity for introducing DNA strand breaks and initiating excision repair [35]. γ -Radiation and EO can cause DNA strand breaks more directly and they induce a short patch type (3–5 nucleotides) of DNA repair synthesis [35,36].

The kinetics of UDS in human lymphocytes induced by UV-radiation, γ -radiation and NA-AAF are well known. γ - and UV-induced UDS have fast components which are essentially completed in 1 h [37] and 1–2.5 h [30,38,39], respectively. On the other hand, NA-AAF-induced UDS has no fast component and is only about 50% completed in 7 h [30,40–42]. The kinetics of EO induced UDS has not been reported, but in our hands the kinetics were quite rapid similar to UV-induced UDS (data not shown).

It has been reported that inhibitors of ADPRT greatly enhance UDS [5,8–11]. Therefore, we thought it of interest to directly compare the effect of ADPRT inhibitors on UDS induced by 4 DNA damaging agents which differ considerably in repair kinetics and endonuclease requirements. The results are reported in Fig. 1. All DNA damaging agents were assayed in resting human mononuclear leukocytes through a dose range which gave maximum stimulation of UDS. Nicotinamide was used at a concentration (2 mM) previously shown to give maximum effects on UDS [9]. γ -, UV-

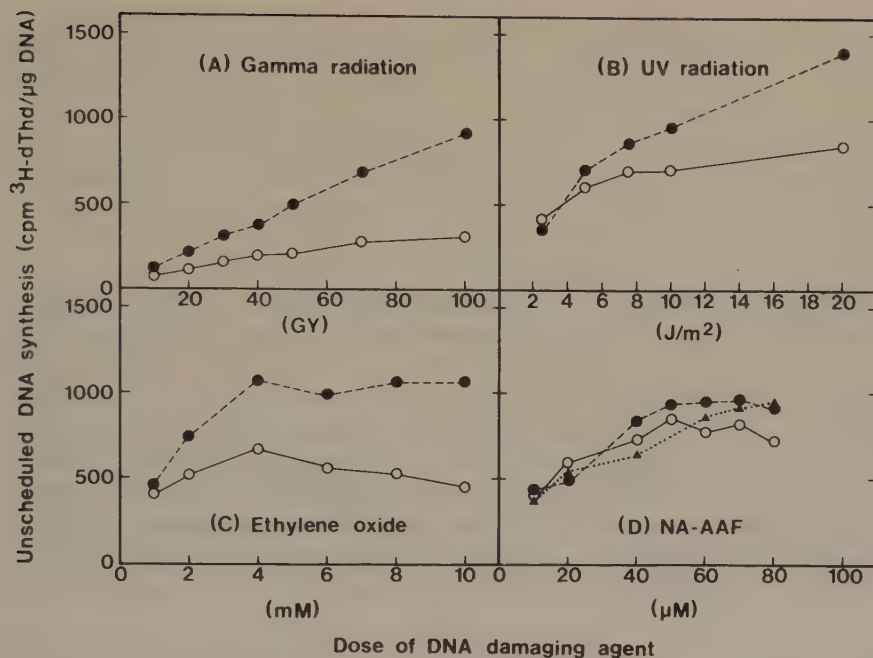


Fig. 1. The effect of inhibitors of ADPRT on UDS induced by various DNA damaging agents in human mononuclear leukocytes. (A) γ -induced UDS with (●—●) and without (○—○) 2 mM nicotinamide; (B) UV-induced UDS with (●—●) and without (○—○) 2 mM nicotinamide; (C) EO-induced UDS with (●—●) and without (○—○) 2 mM nicotinamide; (D) NA-AAF-induced UDS with (●—●) and without (○—○) 2 mM nicotinamide or with (▲...▲) and without (○—○) 2 mM benzamide. Significance levels were calculated from a generalized Student's *t*-test using a test quantity: $t = [\theta^* - E(\theta^*)]/d\theta^*$, where θ^* is the difference between the mean values of UDS with and without ADPRT inhibitor at the different DNA damaging doses, $E\theta^*$ is the expected value of this difference ($\equiv 0$ in the test hypothesis) and $d\theta^*$ the S.E. of θ^* . (A) $P < 0.001$, (B) $P < 0.001$, (C) $P < 0.001$, (D) nicotinamide ($P < 0.05$) and benzamide (not significant).

and EO-induced UDS were all highly significantly increased (i.e. $P < 0.001$, 2-fold increase) in the presence of 2 mM nicotinamide through the dose ranges examined for each of these DNA damaging agents. In contrast NA-AAF induced UDS was not affected by the presence of nicotinamide at doses below 50 μ M NA-AAF and only slightly increased if at all ($P < 0.05$) at the extreme high doses of 50–80 μ M. We interpreted these data to support the hypothesis [22] that the actual number of DNA strand breaks present in the cell, whether introduced directly (e.g. γ -radiation) or induced by excision endonuclease recognition (e.g. UV-radiation or NA-AAF exposure), govern the effects of ADPRT on DNA repair.

Nicotinamide is not only an effective inhibitor of ADPRT, but it is also a direct precursor to NAD⁺ [43]. The cellular pool of NAD⁺ is known to be severely depleted following exposure to DNA damaging agents [44], presumably due to the activation of ADPRT [45]. Therefore, the possibility

existed that we observed no stimulation of NA-AAF induced UDS in the presence of nicotinamide because NA-AAF damage particularly consumed NAD^+ and the added exogenous nicotinamide was functioning mainly as a precursor for NAD^+ biosynthesis. As a result, we have also used another even stronger inhibitor of ADPRT namely benzamide, which has no connection with NAD^+ biosynthesis. It can be seen in Fig. 1 that NA-AAF-induced UDS was also not affected when the repair synthesis was quantified in the presence of 2 mM benzamide.

It has been reported [46–48] that 10 mM hydroxyurea can by itself inhibit the repair of DNA strand breaks. Since we have used 10 mM hydroxyurea in obtaining the data reported in Fig. 1, then our results might have been criticized as relating to our use of such a high concentration of hydroxyurea. In order to resolve this point, we have examined in parallel leukocyte cultures the effects of 2 mM nicotinamide on the level of UDS induced by γ - or UV-radiations, EO and NA-AAF estimated in the presence of both 1 mM and 10 mM hydroxyurea. The results recorded in Table I at 1 mM hydroxyurea were nearly identical to those obtained at 10 mM hydroxyurea; namely, that γ - and UV-radiations and EO caused significant

TABLE I

LACK OF EFFECT OF HYDROXYUREA CONCENTRATION ON THE STIMULATION OF UNSCHEDULED DNA SYNTHESIS BY 2 mM NICOTINAMIDE FOLLOWING INDUCTION OF DNA DAMAGE BY VARIOUS AGENTS IN RESTING HUMAN MONONUCLEAR LEUKOCYTES

Treatment	NA-AAF-induced UDS (cpm [^3H]dThd/ μg DNA)	
	1 mM Hydroxyurea	10 mM Hydroxyurea
(A) 50 Gy γ -radiation	330 $\pm$ 8	307 $\pm$ 15
(B) 50 Gy γ -radiation + 2 mM nicotinamide	441 $\pm$ 31	440 $\pm$ 29
(C) 20 J/m ² UV-radiation	1061 $\pm$ 20	951 $\pm$ 7
(D) 20 J/m ² UV-radiation + 2 mM nicotinamide	1640 $\pm$ 79	1474 $\pm$ 21
(E) 10 mM ethylene oxide	761 $\pm$ 51	625 $\pm$ 23
(F) 10 mM ethylene oxide + 2 mM nicotinamide	1198 $\pm$ 44	1071 $\pm$ 49
(G) 50 μM NA-AAF	1135 $\pm$ 31	1119 $\pm$ 13
(H) 50 μM NA-AAF + 2 mM nicotinamide	1174 $\pm$ 50	1255 $\pm$ 12
<i>t</i> -test	A vs. B = $P < 0.02$ C vs. D = $P < 0.02$ E vs. F = $P < 0.05$ G vs. H = N.S.	A vs. B = $P < 0.02$ C vs. D = $P < 0.002$ E vs. F = $P < 0.02$ G vs. H = N.S.

increases in UDS in the presence of 2 mM nicotinamide but not NA-AAF. Therefore, it was concluded that hydroxyurea did not influence the calculation of UDS induced by the above mentioned genotoxic agents in either the presence or absence of nicotinamide.

The lack of any effect from inhibitors of ADPRT on NA-AAF-induced UDS strongly suggested to us that the enzyme may not be activated by NA-AAF damage during the DNA repair process. In order to study this point, we have estimated ADPRT activity in permeabilized mononuclear leukocytes which had been treated with DNA damaging agents and then allowed to repair in 20% autologous plasma supplemented-physiologic saline for 90 min. We have compared DNA damage induced by γ - and UV-radiations, EO and NA-AAF so that all agents were tested through a dose range in excess of maximum stimulation of UDS (Fig. 1). Figure 2 clearly demonstrates that when γ - and UV-radiation, EO and NA-AAF are compared at DNA damage levels which include data points that result in maximum stimulation of UDS, NA-AAF DNA damage does not give a measureable stimulation of ADPRT activity. On the other hand, γ - and UV-radiations as well as EO all gave highly significant linear dose responses

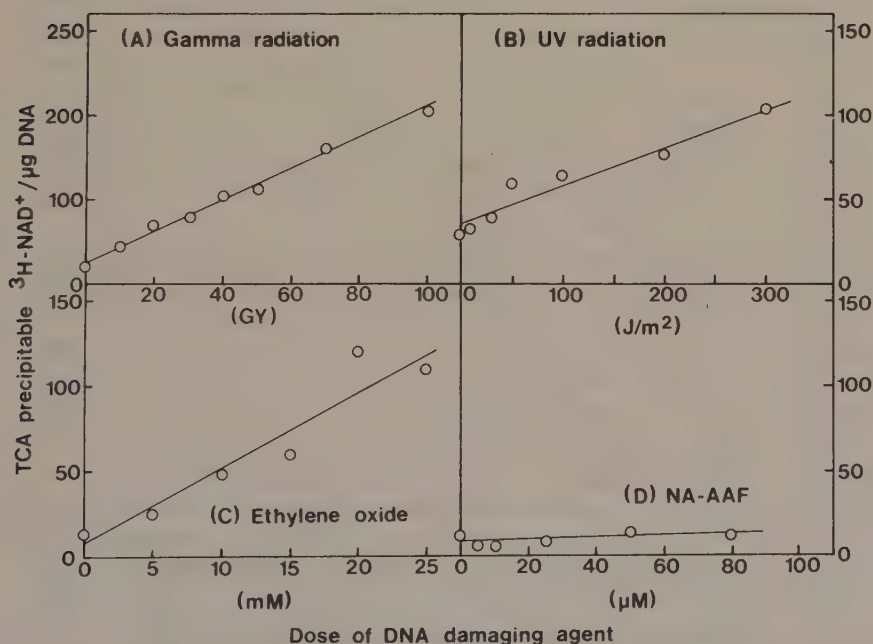


Fig. 2. ADPRT activity in human mononuclear leukocytes following exposure to increasing doses of various DNA damaging agents. The ADPRT activity was expressed as TCA precipitable cpm [^3H]NAD $^+$ per μg DNA that was incorporated into permeabilized cells following DNA damage induction. Linear regression analysis between dose of DNA damaging agent and ADPRT activity gave (A) $Y = 1.8(X) + 26$, $r = 0.99$, $P < 0.001$; (B) $Y = 0.23(X) + 35$, $r = 0.97$, $P < 0.001$; (C) $Y = 4.4(X) + 7$, $r = 0.95$, $P < 0.01$; (D) not significant.

between concentration of these genotoxic agents and stimulation of ADPRT activity. We have interpreted our results to indicate that even when there is so much NA-AAF DNA damage that stimulation of UDS is maximum, there are still so few DNA strand breaks present during the earlier part (i.e. <90 min) of the excision repair process that ADPRT is not stimulated to a higher level of activity.

DISCUSSION

The data recorded in this study are focused on the fact that NA-AAF damage is either not affected by or is extremely insensitive to ADPRT activity. We have used 2 conventional approaches to estimate the effects of the enzyme on DNA repair; i.e. ADPRT inhibitor effects on UDS and direct assessment of enzymatic activation following DNA damage induction. Thus, it is difficult to reason that the lack of any effects from NA-AAF damage on ADPRT activity would be related solely to methodological problems.

The kinetics of repair of NA-AAF induced DNA lesions deserves further comment as NA-AAF differs so strikingly from EO or gamma- and UV-radiations in this regard. We have used a 90 min incubation period, either during or immediately after induction of DNA damage by the 4 genotoxic agents, to allow the mononuclear leukocytes sufficient time to initiate excision repair of the DNA lesions. Since the repair of γ -, UV- and EO-induced DNA lesions have both fast and slow components [30,37-39], the repair of DNA is nearly complete within 90 min. There is no fast component to the repair of NA-AAF-induced DNA lesions [30,40-42], and thus the kinetics of recognizing and removing NA-AAF damage from DNA is a much slower process. Clearly, repair of NA-AAF damage in DNA is taking place as we have observed the induction of UDS (Fig. 1). One working hypothesis to explain the lack of any effects from NA-AAF on ADPRT in mononuclear leukocytes is as follows; i.e., the total number of DNA strand breaks that exist at any time during excision repair synthesis is quite low, because the rate limiting step is the recognition of NA-AAF DNA lesions and excessive breaking by endonuclease activity is minimized. Hence, inhibiting ADPRT has no effect on NA-AAF-induced UDS because there are so few DNA strand breaks present at any one time that the enzyme does not get activated to a higher level, and thus, ADPRT inhibitors have no effect on NA-AAF-induced UDS. Support for this hypothesis is the fact that NA-AAF-induced DNA damage does not enhance ADPRT activity in mononuclear leukocytes even at very high NA-AAF doses (Fig. 2).

Nevertheless, one important point has emerged from this study; i.e. UDS induced by NA-AAF damage involves only minimally or not at all ADPRT. The UDS induced by other agents such as UV-radiation quantify not only the repair synthesis induced by the UV-radiation but also that portion of repair synthesis which is regulated by the stimulation of ADPRT (Figs. 1 and 2). We, therefore, feel that studies of interindividual variations in

DNA repair capacity, where the estimations were based mainly on UV induced UDS [31,32,49–54], may suffer from poor quantification of the DNA polymerase step in excision repair because the level of UDS has also been influenced by activation of ADPRT (Fig. 2 and Table I).

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Effects of γ Radiation and Hyperthermia on DNA Repair Synthesis and the Level of NAD^+ in Cultured Human Mononuclear Leukocytes

GÖRAN G. JONSSON,*† GÖRAN ERIKSSON,‡ AND RONALD W. PERO†

*Department of Oncology, University Hospital, Lund, Sweden; ‡ Department of Pathology, University Hospital, Lund, Sweden; and †Biochemical and Genetic Ecotoxicology, Wallenberg Laboratory, University of Lund, Lund, Sweden

JONSSON, G. G., ERIKSSON, G., AND PERO, R. W. Effects of γ Radiation and Hyperthermia on DNA Repair Synthesis and the Level of NAD^+ in Cultured Human Mononuclear Leukocytes. *Radiat. Res.* 97, 97-107 (1984).

DNA repair has been investigated, estimated by unscheduled DNA synthesis (UDS) and the cellular NAD^+ pool, after exposing human mononuclear leukocytes to hyperthermia and γ radiation separately and in combination. It was found that γ radiation induced a decline in UDS with increasing temperature through the temperature region studied (37-45°C). At 42.5°C the γ -ray-induced UDS was reduced to about 70% of that at 37°C. Following γ -ray damage the NAD^+ pool dropped to about 20% of control values. Without hyperthermic treatment the cells completely recovered to the original level within 5 hr. Moderate hyperthermia (42.5°C for 45 min) followed by γ -ray damage altered the kinetics so that even after 8 hr the NAD^+ pool had recovered to only 70% of the original level. After heat treatment at 44°C for 45 min prior to γ radiation the cells did not recover at all, presumably because of the cytotoxic effects from the combined treatment.

INTRODUCTION

Substantial evidence for cytotoxic effects on tumor cells after a combined treatment with hyperthermia and ionizing radiation has accumulated (1-4), and such a treatment is becoming a potentially useful tool in cancer therapy.

It is known that well-oxygenated cells or tissues are much more sensitive to ionizing radiation than when they are hypoxic (5-7). In living tissue hyperthermia may induce physiological modifications. Bicher *et al.* (8) have found at 41°C an increased tissue oxygen tension following increased micro blood flow, thereby increasing the radio-sensitivity of tumors. At higher temperatures the micro blood flow eventually collapses, resulting in lower oxygen tension and further reduction of the already low tumor pH which, by itself, may be more lethal for tumor than for normal tissue.

Furthermore, it is known that tissues which have a large portion of their cells in the DNA synthetic phase of the cell cycle (e.g., tumor cells) are more sensitive to hyperthermia than tissues with cells in other phases (9-12).

Taken together these data support the hypothesis that hyperthermia in combination with ionizing radiation may be used to selectively destroy neoplastic cells while sparing the less sensitive normal tissue.

One effect of hyperthermia when combined with DNA damaging agents is a decreased rate of repair of DNA strand breaks (13–20). The molecular basis of these findings is by no means clear, and several hypotheses have been suggested: (i) increased efficiency of production of DNA damage, (ii) inhibition of enzymatic repair, (iii) partial denaturation of DNA, histones, or other nucleoproteins, (iv) effects on RNA metabolism, (v) altered cellular membranes, and (vi) effects on lysosomal activity [see review by Roti Roti (21)].

Warters and Roti Roti (17, 20) have suggested that changes in the chromatin structure, rather than enzyme denaturation, reduce the repair rate in γ -irradiated cells after hyperthermic exposure above 43°C. There is at least one enzyme which is known to alter chromatin structure during repair, poly(ADP-ribose)polymerase. This chromosomal enzyme catalyzes the polymerization of ADP-ribose moieties derived from NAD^+ to chromatin proteins including histones (22). Several studies now indicate that this enzyme has a role in cellular repair of DNA damage (23–34).

DNA-damaging agents are known to induce the activity of poly(ADP-ribose)polymerase, thereby depleting the NAD^+ pool, which later recovers (25). The apparent role of NAD^+ in cell metabolism and DNA repair has stimulated us to look for a possible effect of hyperthermia on the cellular NAD^+ pool in connection with DNA damage induced by γ radiation.

In this paper we have investigated unscheduled DNA synthesis (UDS), cellular NAD^+ levels, and ATP, ADP, and AMP pools in human mononuclear leukocytes following exposure to hyperthermia and γ radiation separately and in combination. Our results indicate that hyperthermia and γ radiation interact synergistically by inhibiting the recovery of depleted cellular NAD^+ levels.

MATERIALS AND METHODS

Isolation of mononuclear leukocytes. Human venous blood was collected in heparinized vacutainers (143 USP units/tube). The platelet rich plasma was removed by centrifugation at 100g for 10 min and physiologic saline solution was added to the original volume. Thirty milliliters of the suspension were carefully layered on 15 ml Isopaque-Ficoll solution (Lymphoprep, Nyegaard and Co.). After centrifugation at 400g for 30 min, the mononuclear blood cells were collected from the interphase, and the plasma was liberated from the platelets. The mononuclear leukocytes were washed in saline solution and counted, then resuspended in Eagle's MEM with Hank's salts (Gibco). More than 90% of the platelets are removed when the cells are prepared in this manner. Cultures contained about 15×10^6 cells in 4 ml of this medium plus 1 ml autologous plasma, 2.5 mM L-glutamine, penicillin (50 $\mu\text{g}/\text{ml}$), and streptomycin (50 $\mu\text{g}/\text{ml}$). Cultures were prepared at room temperature.

Hyperthermia treatment and γ irradiation. In all experiments hyperthermia was given prior to radiation. The cells were placed in water baths for 45 min at temperatures varying from 39.5 to 45°C and in parallel to control samples at 37°C for the same time. The temperatures were measured with an accuracy of about $\pm 0.2^\circ\text{C}$. The heat treatment period of 45 min was chosen because that time is used in clinical application of hyperthermia and radiation in our hospital (4).

Immediately after the hyperthermia treatment the cell cultures were put on ice to retard the DNA repair process and irradiated in a ^{137}Cs γ -ray source (Scandiatronix "Gammaraad 900", 0.8 Gy/min) parallel to controls on ice. After irradiation the samples for UDS measurements were incubated for 18 hr at 37°C. DNA repair following γ -ray damage contains a fast component and a slower step (35). We have used an 18-hr pulsing time with [^3H]thymidine present as a matter of convenience (36) as well as in an effort to better quantify the slow DNA repair component. Samples for NAD^+ pool measurements were incubated for various times from 15 min up to 8 hr at 37°C. Pilot experiments showed that the samples equilibrated to the desired temperature within 30 min.

Measurements of UDS. For the radiation-induced repair synthesis measurements 10 mM hydroxyurea (Sigma) were added immediately after the preparation of the cultures and [^3H]thymidine (10 $\mu\text{Ci/ml}$, 25 Ci/mole, Amersham) was added immediately after the irradiation step. Hydroxyurea is used to suppress incorporation of [^3H]thymidine due to ordinary replicative DNA synthesis. After 18 hr repair the cells were harvested by centrifugation at 300g for 10 min. The DNA was extracted and purified, and radioactivity was counted and quantitated by the method described previously (36). The results were recorded as cpm [^3H]thymidine incorporated per microgram DNA.

Measurement of NAD^+ , ATP, ADP, and AMP pools. After the appropriate incubation times the cells were harvested by centrifugation for 5 min at 300g and $+20^\circ\text{C}$. Each sample was suspended in 1.8 ml physiologic saline and transferred to 2-ml glass homogenizers, and 25- μl aliquots were taken for manual cell counting. The cells were spun down for 30 sec at 2000g and $+4^\circ\text{C}$, and the supernatant was discarded. The pellets in the homogenizers were frozen in liquid nitrogen and stored at -80°C .

The day after preparation the homogenizers were brought to a cryostat (Linde) cooled to -20°C . To the frozen pellets 10 μl of a solution composed of 0.70 M perchloric acid, 30% (v/v) methanol, and 0.5 mM nicotinic acid were added per 10^6 cells. The pellets were loosened and homogenized for 3 min. The homogenizers with the pestles were centrifuged for 30 sec at 150g and -20°C . The pestles were removed and the extracts were centrifuged again for 1 min at 12,000g. The tubes were put into the cryostat. Aliquots of the supernatants were transferred to 1.5-ml Eppendorf tubes containing a mixture of 150 μl 7 M KOH, 200 μl 0.4 M triethanolamine buffered with HCl to pH 7.2, and 50 μl thymol blue (3 mg/10 ml 8 mM KOH). The ratio of the mixture to the aliquot of the supernatant was 1:5. The tubes were stirred, 2 M KOH was added until the color changed from red to yellow, and then the solution was carefully adjusted to pH 6.6–7.0 as determined with indicator paper (pH 6.4–8.0, Merck). The extracts were then centrifuged for 1 min at 44,000g and -20°C , and the supernatants were transferred to Eppendorf tubes and stored at -80°C .

Isotachopheresis was performed on an LKB 2127 Tachophor with an electrolyte system as described previously (37). To 4 μl cell extract, 1 μl of the following spacer solution was used to increase separation: 0.3 mM phosphoenolpyruvic acid, 0.3 mM phosphoglyceric acid, 0.4 mM chloroacetic acid, 0.3 mM trichloroacetic acid, 0.05 mM CTP, 0.3 mM glucaric acid, 0.4 mM glyceric acid, 0.3 mM acetic acid, 0.4 mM levulinic acid, and 0.8 mM propionic acid. Nicotinic acid added at the extraction was used as an internal standard to calculate the dilution constant. Concentrations of different substances could then be determined per 10^6 cells.

Estimation of viabilities. After the appropriate incubation times aliquots were removed and mixed with an equal volume or 0.4% trypan blue dissolved in Ringer's solution. The cell suspensions were incubated at 37°C for 30 min and immediately placed on ice. The percentage of unstained (viable) cells was then determined by light microscopy.

RESULTS

γ -Ray-Induced UDS and Hyperthermia

For this study we used four blood donors who showed the same dependence of UDS on temperature although there were some individual variations in the levels of UDS (36). The data from one individual are given in Fig. 1, which shows the level of UDS following damage of the mononuclear leukocytes by 30-Gy γ rays versus temperature with the hyperthermic treatment immediately before irradiation. UDS declined with increasing temperature as evidenced by a highly significant linear regression correlation coefficient between temperature and UDS of $r = -0.91$ ($P < 0.001$). The average data for all four individuals gave the same correlation ($r = -0.90$, $P < 0.001$). At 42.5°C the UDS level was about 30% lower than at the control temperature of 37°C .

The level of hydroxyurea-suppressed replicative synthesis showed a slight decrease with increasing temperature; however, it was not statistically significant.

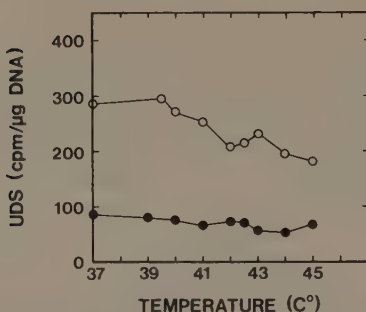


FIG. 1. Unscheduled incorporation of [^3H]dThd following DNA damage by 30-Gy γ radiation as a function of heat treatments for 45 min given immediately prior to the radiation (open circles). Filled circles show the corresponding hydroxyurea-suppressed replicative synthesis.

NAD⁺ Pools after γ Radiation and Hyperthermia

A typical nucleotide profile is given in Fig. 2. The various nucleotides were identified using criteria previously described in detail (37). We utilized freshly isolated mononuclear leukocytes from 18 blood donors. The NAD⁺ levels varied for these individuals from 78 to 144 pmole/ 10^6 cells with an average value of 118 ± 17 pmole/ 10^6 cells (mean $\pm$ SD). Berger *et al.* (38) found for 20 blood donors NAD⁺ levels between 40 and 114 pmole/ 10^6 cells.

Figure 3 shows NAD⁺ levels versus incubation time at 37°C following 30-Gy radiation exposure and hyperthermia treatment for 45 min at 37 (controls), 42.5, and 44°C prior to radiation. Hyperthermia exposure itself did not alter the NAD⁺ pool outside the reproducibility range of our method, being about $\pm 5\%$. However, cells treated at 44°C showed a slight reduction of the NAD⁺ level for incubation times longer than 5 hr (data not shown).

Damaging the mononuclear leukocytes with γ radiation dramatically depleted the NAD⁺ pool to less than 20% of the original value. The NAD⁺ pool was completely restored within 5 hr when the cells were incubated at the control temperature of 37°C prior to γ radiation.

When a heat shock of 42.5°C was given prior to γ radiation, the recovery time was about the same, but the NAD⁺ level was restored to only 70% after 8 hr.

Heat treatment at 44°C for 45 min prior to γ radiation resulted in a complete loss of recovery, and the NAD⁺ pool was only 20% of the original value even after 8 hr of incubation.

ATP, ADP, and AMP Pools after Hyperthermia and γ Radiation

Data for the ATP, ADP, and AMP levels from the 18 blood donors are given in Table I.

Figures 4a–c show the ATP levels in relative scale versus incubation time following exposure of the cells to heat alone (open circles) or given prior to 30-Gy γ radiation (filled circles). The hyperthermia temperatures were 37, 42.5, and 44°C, and treatments lasted for 45 min.

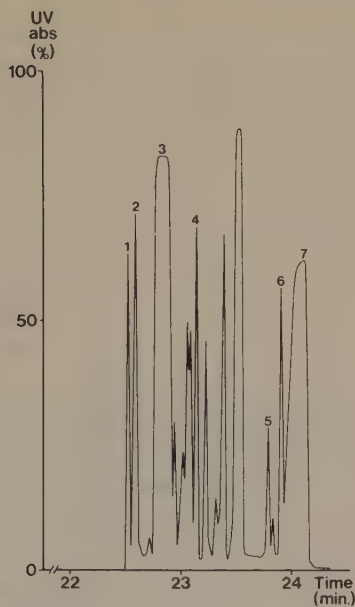


FIG. 2. Nucleotide profile from freshly isolated human mononuclear leukocytes (recorded at 254 nm). 1: UTP, 2: GTP, 3: ATP, 4: ADP, 5: AMP, 6: NAD^+ , 7: nicotinic acid (internal standard).

When the cells were exposed to 30-Gy γ radiation after heat treatment, we found a reduction of ATP; the depletion was larger the higher the temperature. At lower temperatures the ATP levels began to approach those of the unirradiated samples. However, at 44°C the cells were unable to restore their ATP levels completely even after 8 hr of incubation.

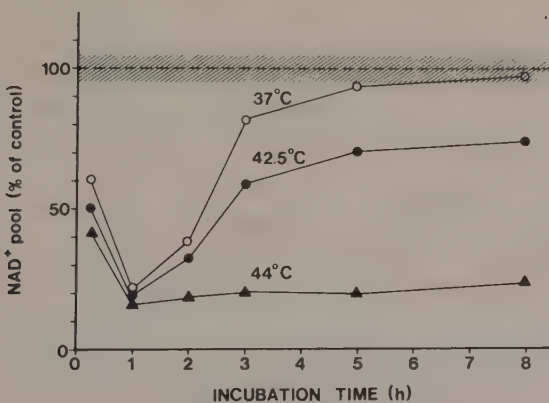


FIG. 3. NAD^+ levels relative to untreated controls versus incubation time at 37°C following hyperthermia and γ radiation. The leukocytes were given heat treatments at 37 (controls), 42.5, and 44°C for 45 min prior to 30-Gy γ radiation. The hatched area shows the range of values for unirradiated controls at 37, 42.5, and 44°C.

TABLE I
Adenosine Nucleotide Concentrations in Normal Freshly Isolated Human Mononuclear Leukocytes (*n* = 18)^a

	<i>Min.</i>	<i>Max.</i>	<i>Mean</i> ± <i>SD</i>
ATP	897	1298	1140 ± 188
ADP	145	316	211 ± 46
AMP	58	95	69 ± 11

^a Values in pmole/10⁶ cells.

Figures 5a–c show the corresponding ATP/ADP ratios in relative units versus incubation time. The ATP/ADP ratios following combined treatment of hyperthermia and γ radiation decreased with increasing temperature, thus reflecting an increasing lack of balance between energy utilization and energy production.

Figure 6 shows the synergistic effect of a heat shock at 44°C and 30-Gy γ radiation on ATP, ADP, and AMP pools relative to cells exposed to hyperthermia alone. The ATP and ADP pools dropped considerably with only a slight trend toward recovery, whereas the corresponding AMP pool typically increased in response to depletion of ATP and ADP. The forms of the three curves reflect the metabolic state of energy production and utilization in the damaged cells.

The nucleoside triphosphates UTP and GTP could also be determined from the isotachophor profiles (Fig. 2) and were found to fluctuate in a manner similar to the ATP pool (data not shown).

Effects of γ Radiation and Hyperthermia on Cell Viability

Since mononuclear leukocytes are mainly in G0 phase of the cell cycle, we have used trypan blue exclusion to estimate cell survival instead of other techniques that require DNA synthesis.

Figure 7 shows viability of the cells versus incubation time for the various treatments. Hyperthermia treatment at either 42.5 or 44°C for 45 min did not in itself induce cell death within the first 10 hr of incubation. This was an important control to establish that reduced pools observed over an 8-hr incubation period were not due

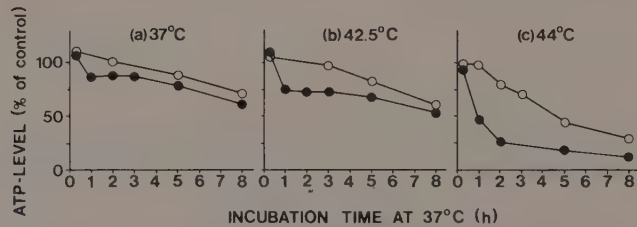


FIG. 4. ATP levels relative to untreated controls versus incubation time at 37°C. Open circles show ATP levels following hyperthermia treatment alone: (a) 37°C, (b) 42.5°C, (c) 44°C. Filled circles show ATP levels following hyperthermia at these temperatures plus 30-Gy γ radiation prior to the heat shock.

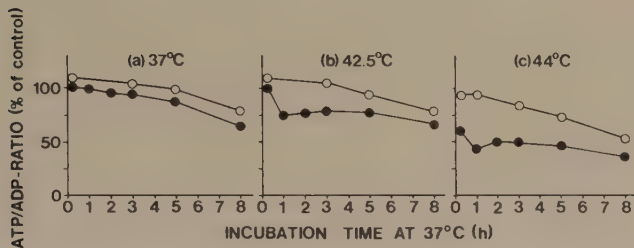


FIG. 5. ATP/ADP ratios relative to untreated controls versus incubation time at 37°C. Symbols as in Fig. 4.

to cell necrosis induced by hyperthermic shock alone. In addition hyperthermia in combination with 30-Gy γ radiation clearly increased the cytotoxic action of either agent administered separately (Fig. 7). This was true even in the absence of any appreciable DNA synthesis which suggests that nucleotide pool imbalance may affect cell survival directly.

DISCUSSION

One of the most promising molecular models proposed to explain the enhancement of cell toxicity induced by γ radiation when combined with hyperthermic shock has centered around the hypothesis that hyperthermia inhibits the enzymatic-mediated repair of γ -ray-induced DNA lesions (16-20). We have shown that a combined treatment of hyperthermia and 30-Gy γ radiation significantly reduces UDS in mononuclear leukocytes over those treated with 30-Gy γ radiation. This reduction can be ascribed

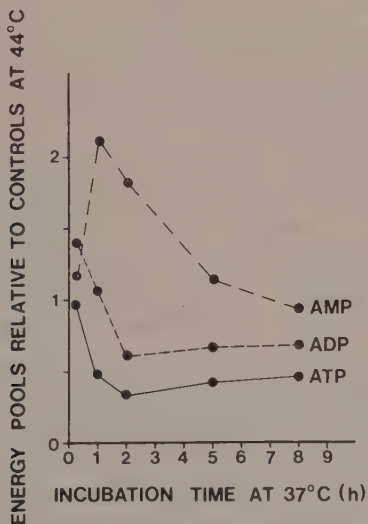


FIG. 6. ATP, ADP, and AMP levels at 44°C for 45-min 30-Gy γ radiation relative to controls at 44°C as a function of incubation time. All data points have been divided by the values obtained after hyperthermia treatment of 44°C for 45 min only.

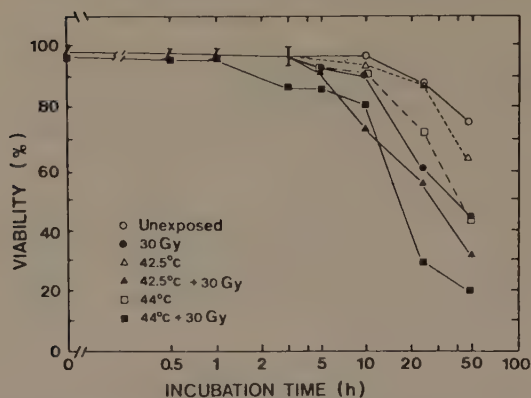


FIG. 7. Viability versus incubation time at 37°C after the various treatments. At short incubation times the viabilities were 97–100% in all treatments and those data are therefore indicated by bars.

to several processes regulating DNA repair, e.g., rate-limiting effects on DNA glycosylase and exonuclease activities, the nucleotide pool, and the DNA polymerase itself, which in fact has been reported (39).

The exact role of poly(ADP-ribose)polymerase in the excision repair process is not known. Both endonuclease (23) and ligase (40) are ADP-ribosylated, which in turn alters the capacities of these enzymes to repair DNA. It has also been reported (41, 42) that γ radiation induces poly(ADP-ribose)polymerase activity but DNA repair of these lesions can proceed even in the presence of inhibitors for this enzyme.

In addition to DNA-damaging agents, cell differentiation (43, 44) and gene expression (45) also induce DNA strand breakage and thereby activate poly(ADP-ribose)polymerase. Apparently the steady-state level of DNA strand breaks (42), which is under potential control via regulation of endonuclease or ligase activities by ADP-ribosylation, is crucial for involving poly(ADP-ribose)polymerase directly in the DNA repair process.

The main purpose of this study was to examine a model cell system and, at the molecular level, the effects of hyperthermia and γ radiation in killing tumor cells *in vitro*. In clinical practice, high doses of γ radiation are commonly used [i.e., >30 Gy, Refs. (1–4)]. This is the reason we have used such high doses of γ radiation in this study. Under these conditions, the cells are given a vast excess of strand breaks to repair immediately, and thus the rate-limiting step is likely to be a repair enzymatic activity (e.g., ligase) which can be further activated to a higher level by ADP-ribosylation. This is supported by data regarding the cytotoxic effects of γ radiation and inhibitors of poly(ADP-ribose)polymerase activity. A γ -radiation dose 0.4–1 Gy in the presence of an inhibitor of poly(ADP-ribose)polymerase was shown to reduce cell survival slightly but consistently (46). However, γ -radiation doses <0.4 Gy in the presence of the same enzyme inhibitor did not reduce cytotoxicity over the effects of γ radiation alone (42). In this study, when 30 Gy of γ radiation plus hyperthermia of 42.5°C for 45 min were used (i.e., conditions that prevented NAD⁺ pool regeneration), cell survival was distinctly reduced compared to that following 30-Gy γ

radiation alone (Fig. 7). In other words, when the demand for NAD^+ is great because of excessive DNA strand breakage, then the function of poly(ADP-ribose)polymerase becomes critical to increase DNA repair capacity and reduce cytotoxicity. One explanation can be that, if the NAD^+ depletion is not restored by other metabolic events such as ATP production (Fig. 4), then the function of poly(ADP-ribose)polymerase may become reduced because of lack of the co-substrate NAD^+ .

Hyperthermia alone induces tumor regression [see, e.g., Ref. (47)], although we have observed no effects from hyperthermia treatment alone on UDS (i.e., DNA polymerase activity, Fig. 1) or NAD^+ pools [i.e., poly(ADP-ribose)polymerase activity, Fig. 3]. On the other hand, we have shown that the energy pool balance reflected by the ATP/ADP ratios was reduced by hyperthermic treatment alone at 44°C for 45 min (Fig. 5), and other investigators (48) have shown that ATP deprivation enhances cell killing by hyperthermia alone. Hence one hypothesis is that a depletion of ATP from hyperthermia causes a limited generation of NAD^+ (48), which in turn can inhibit repair after γ -ray-induced DNA damage via the reduced amount of donor substrate for the NAD^+ consuming enzyme, poly(ADP-ribose)polymerase. This hypothesis is under investigation in our laboratory.

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Influence of Hyperthermia and γ Radiation on ADP-Ribosyl Transferase, NAD^+ , and ATP Pools in Human Mononuclear Leukocytes

GÖRAN G. JONSSON,*[‡] GÖRAN ERIKSSON,[†] AND RONALD W. PERO[‡]

*Department of Oncology, and [†]Department of Pathology, University Hospital, Lund, Sweden
[‡]Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Lund, Sweden

JONSSON, G. G., ERIKSSON, G., AND PERO, R. W. Influence of Hyperthermia and γ Radiation on ADP-Ribosyl Transferase, NAD^+ , and ATP Pools in Human Mononuclear Leukocytes. *Radiat. Res.* 102, 000-000 (1985).

Effects of hyperthermia (42.5°C) and γ radiation (30 Gy) on ADP-ribosyl transferase, NAD^+ , and ATP pools in human mononuclear leukocytes have been investigated. It was found that the γ -ray activation level of the enzyme was not influenced by this hyperthermia for 45 min. Following deprivation of ATP synthesis by 2,4-dinitrophenol, an uncoupler of the oxidative phosphorylation, and omitting glucose from the culture medium, the NAD^+ pool was reduced to about 60% of control value. The potentiation of ATP production by exogenously supplied adenosine was reduced after a combined treatment of the cells with hyperthermia and γ radiation. Mitochondrial and endoplasmic changes within the mononuclear leukocytes were also observed. Based on these findings a model for the hyperthermia effect is proposed. © 1985

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INTRODUCTION

Cells exposed to genotoxic chemical agents or γ or UV radiation immediately start repair processes that involve excision of DNA lesions, new DNA synthesis, and ligation of the repaired patch of DNA. These processes are known to require various precursor and cofactor substances including nucleosides, ATP, and NAD^+ (1, 2).

When considering cancer therapy it is of great interest to find methods that increase the cytotoxicity of, for example, γ radiation. Hence it would seem quite logical to interfere with these DNA repair processes and make them less successful provided that tumor cells could be preferentially killed and normal tissue spared.

One approach has been to use supranormal temperatures, i.e., hyperthermia. Hyperthermia and γ radiation have been shown to interact in a synergistic way to induce a greater cytotoxicity than can be achieved by summing up the effects of hyperthermia and radiation alone; i.e., hyperthermic radiosensitization (3, 4).

Cells are most sensitive to radiation at or close to mitosis, whereas the cells that are most sensitive to the cytotoxic effects of hyperthermic treatment are in late S phase. On the other hand, cells in late S phase are also most radioresistant (5-7).

Several hypotheses for the sensitizing cytotoxic effects of hyperthermia have been suggested including (i) increased efficiency of producing DNA damage, (ii) inhibition

of enzymatic repair, (iii) partial denaturation of nuclear proteins, and (iv) membrane alterations (for Ref. see (8)). However, as yet none have been fully substantiated.

We have shown in a previous paper (9) that hyperthermia in combination with γ radiation carried out on human mononuclear leukocytes prevented regeneration of NAD^+ and delayed ATP regeneration. When cells were given γ radiation alone, the NAD^+ pools were completely restored within about 5 hr. These data have suggested to us still another possible hypothesis for the mechanism of radiosensitization by hyperthermia.

Firstly, adenosine diphosphate ribosyl transferase (ADPRT), which is chromatin bound, is known to be activated by genotoxic agents that induce DNA strand breaks, and since NAD^+ is a substrate for ADPRT then cellular NAD^+ pools are depleted (10, 11). It has been suggested that ADPRT has a function in DNA repair (2, 12–14) by affecting the endonuclease activity (15, 16) and/or ligase activity (17) via ADP-ribosylation of these enzymes. Moreover, inhibition of ADPRT has been found to stimulate repair (18–21) and to increase the cytotoxicity of DNA damaging agents (12–14).

Secondly, hyperthermia may directly alter ADPRT activity which has in fact been found for *Drosophila* cells (22), and it may also interfere with the regeneration of NAD^+ since this biochemical process has ATP-dependent steps (23, 24). ATP may be directly involved in DNA repair (25–27), but certainly it is involved indirectly because it, e.g., reacts with the NAD^+ precursor nicotinamide mononucleotide in a condensation reaction to yield NAD^+ . Here we report on testing the hypothesis that hyperthermia radiosensitizes because it limits the supply of NAD^+ which, in turn, is needed as substrate for ADPRT during the repair of DNA damage induced by γ radiation.

MATERIALS AND METHODS

Isolation of mononuclear leukocytes. Blood samples were taken from apparently healthy donors by venous puncture and collected in heparinized vacutainers (143 U.S.P. units/tube). The mononuclear leukocytes were isolated by centrifugation on an Isopaque-Ficoll gradient (Lymphoprep, Nyegaard and Co.) as described earlier by us (9).

Activity of ADP-ribosyl transferase. Cultures containing about 10×10^6 mononuclear leukocytes in 4 ml 0.9% NaCl plus 1 ml autologous platelet poor plasma were heated in water baths for 45 min at 42.5°C and then irradiated on ice to 30 Gy in a ^{137}Cs γ -ray source (Scandiatronix "Gammarad 900," dose rate 0.8 Gy/min). Controls were kept at 37°C for 45 min before irradiation. After treatment the cells were incubated for various times at 37°C and harvested by centrifugation at 300g for 10 min. The cells were permeabilized in hypotonic buffer, and then tritium-labeled NAD^+ (i.e., 25 μM , [^3H]adenine, 150 mCi/mmole) and reaction buffer were added using the procedure by Berger (28) with minor modifications as described earlier by us (29). The data were recorded as cpm [^3H] NAD^+ incorporated into TCA precipitable material per μg DNA.

Determination of NAD^+ and ATP pools. After treatment the cells were incubated at 37°C for 5 hr and then harvested by centrifugation at 300g for 10 min. Each sample was suspended in 1.8 ml physiologic saline and transferred to 2-ml glass homogenizers and 25- μl aliquots were taken out for automatic cell counting (Coulter counter, Coulter Electronics). The cells were spun down at 2000g for 30 sec, the supernatant was discarded, and the pellets in the homogenizers were frozen in liquid nitrogen and stored until the next day at -80°C .

The frozen cell pellets were extracted with cold perchloric acid and methanol as described previously (9). The neutralized supernatant was analyzed on an LKB 2127 Tachophor. The principle of isotachopheresis is based on migration of ions in an electric field with all ions moving at the same velocity (see, e.g.,

review in Ref. (30)). Utilization of intermediate mobile compounds with effective ion mobilities between those of the nucleotides to be separated (so-called spacers) results in UV absorbance spectra with discrete peaks whose areas correspond to the quantity of nucleotides (9). The isotachophoretic separations were done either with the standard equipment (9) or with an extended column-coupling system.¹ With this system an increased volume (10 μ l) could be injected giving more information and decreasing the number of spacers needed. As a result, 1 μ l of the following spacer solution gives similar separation as the standard separation method; 0.11 mM phosphoenolpyruvic acid, 0.4 mM trichloroacetic acid, 0.4 mM chloroacetic acid, and 0.3 mM glucaric acid. The nucleotide pools were recorded as picomoles per 10⁶ cells.

Experiment with uncoupler, hyperthermia, and γ radiation. Cultures containing about 15×10^6 cells were prepared in two different culture media containing (a) 4 ml physiologic saline plus 1 ml autologous plasma plus 1 mM glucose and (b) 4 ml physiologic saline plus 1 ml plasma. Hyperthermia of 42.5°C for 45 min was given immediately before radiation with and without 100 μ M 2,4 dinitrophenol (DNP) (Sigma) present in the culture media; controls were treated similarly at 37°C. Immediately after heat treatment the cells were put on ice and irradiated to 30 Gy parallel to control samples on ice. After the irradiation the samples were incubated for 5 hr at 37°C and then the nucleotide pools were measured by isotachopheresis as described above¹ (9).

Experiment with adenosine, hyperthermia, and γ radiation. These cultures were arranged to contain about 15×10^6 cells in 4 ml Hanks' Eagle (GIBCO) plus 1 ml horse serum, 2.5 mM L-glutamine, penicillin (50 μ g/ml), and streptomycin (50 μ g/ml). After preparation and heating the samples were immediately put on ice for irradiations as above. Immediately after the irradiation adenosine in various concentrations up to 500 μ M plus 1 mM uridine were added and the samples were incubated for 5 hr at 37°C. The nucleotide pools were then measured as we have described above¹ (9).

Electron microscopy. Cultures containing 10×10^6 mononuclear leukocytes in 4 ml Hanks' Eagle (GIBCO) plus 1 ml autologous plasma (platelet poor) with antibiotics present were heated to 42.5°C and controls at 37°C for 45 min. The cells were then put on ice and irradiated to 30 Gy. After a 5-hr repair incubation at 37°C the cells were spun down at 300g for 10 min. The pellets were fixed for 8 hr in 2% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose. After rinsing in cacodylate buffer the pellets were postfixed for 1 hr in OsO₄, dehydrated in graded series of alcohol, embedded in Agar Resin 100, cut in an ultra microtome (LKB), and contrasted with uranyl acetate plus lead citrate (Reynolds classical double stain). The cells were photographed in a transmission electron microscope (Zeiss EM 10).

RESULTS

ADP-ribosyl transferase activity after hyperthermia and γ radiation. The enzyme activity measured is shown in Fig. 1. Hyperthermia of 42.5°C for 45 min did not change the enzyme activity from control levels. Cells damaged with 30 Gy γ radiation strongly activated the enzyme. This activation level was not significantly altered within the experimental accuracy of our method (about 10%) when hyperthermia was given to the cells prior to the radiation (Student's *t* test). During the 5-hr interval in which we incubated the cells to allow initiation of DNA repair, the levels of γ -ray-induced ADPRT remained elevated. These data support the theory that the cells were given a dose of γ radiation (30 Gy) that saturated ADPRT activity.

Uncoupler, hyperthermia, and γ radiation. Our data are shown in Table I. We have compared the NAD⁺ and ATP pools of controls held at 37°C, after hyperthermia at 42.5°C for 45 min, and after 30 Gy γ radiation with and without 100 μ M dinitrophenol (DNP) to test effects of uncoupled oxidative phosphorylation in the presence and absence of glucose-supplemented culture media. Even in the presence of uncoupler some ATP production can take place by the route of glycolysis in the

¹ G. Eriksson, Thesis, Department of Pathology, University Hospital, Lund, Sweden, 1983

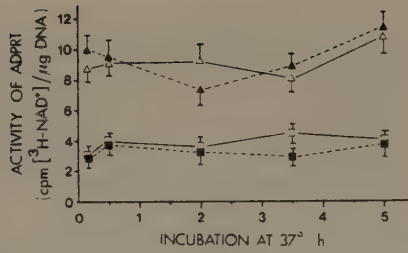


FIG. 1. Activity of ADPRT versus incubation time at 37°C after various treatments. □ controls at 37°C, ■ 42.5°C for 45 min, △ 30 Gy γ radiation, ▲ 42.5°C plus 30 Gy. For experimental details see Materials and Methods. Error bars indicate the standard deviations.

presence of glucose. The cells deprived of ATP by treatment with DNP during and after γ radiation did not have reduced NAD^+ pools unless glucose was omitted from the culture medium. DNP does not affect ATP production from glycolysis, and γ radiation itself did not interfere with ATP production from glycolysis which was sufficient to prevent depletion of ATP and NAD^+ in the presence of uncoupler. However, ATP and NAD^+ pools were depleted after hyperthermia in the presence of DNP even with cells cultured in glucose-supplemented media (compared to

TABLE I
ATP and NAD^+ Pools following Treatment with γ Radiation and Hyperthermia

Treatment	ATP (pmole/ 10^6 cells)			NAD^+ (pmole/ 10^6 cells)		
	SPG ^a	SP ^b	t-test SPG vs SP	SPG	SP	t test SPG vs SP
(A) 37°C - DNP ^c	1083 $\pm$ 100	907 $\pm$ 90	NS	153 $\pm$ 15	120 $\pm$ 10	NS
(B) 37°C + DNP	800 $\pm$ 80	773 $\pm$ 80	NS	126 $\pm$ 12	117 $\pm$ 10	NS
t test A vs B	NS	$P < 0.1$		NS	NS	
(C) 42.5°C - DNP	813 $\pm$ 80	ND ^d	—	112 $\pm$ 10	ND	—
(D) 42.5°C + DNP	493 $\pm$ 50	ND	—	72 $\pm$ 7	ND	—
t test C vs D	$P < 0.05$	—		$P < 0.05$	—	
(E) 30 Gy - DNP	730 $\pm$ 70	670 $\pm$ 70	NS	115 $\pm$ 12	100 $\pm$ 10	NS
(F) 30 Gy + DNP	700 $\pm$ 70	576 $\pm$ 60	$P < 0.05$	112 $\pm$ 12	73 $\pm$ 7	$P < 0.01$
t test E vs F	NS	$P < 0.1$		NS	$P < 0.01$	

Note. The table summarizes data from five different blood donors, standard deviation being about 10%. Treatments (C) and (D) were carried out using Hanks' Eagle medium in the place of saline. These conditions gave comparable results but they prohibit statistical comparisons to treatments (A), (B), (E), and (F). Human mononuclear leukocytes were cultured in media containing varying amounts of glucose. The pools were measured after a 5-hr repair incubation at 37°C in the presence and absence of 100 μM 2,4 dinitrophenol.

^a SPG = Saline + autologous plasma (20%) + glucose (1 mM).

^b SP = Saline + autologous plasma (20%).

^c DNP = 2,4 dinitrophenol (100 μM).

^d ND = Not determined.

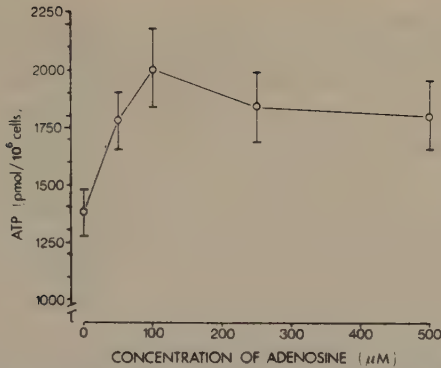


FIG. 2. ATP pool versus concentration of adenosine in culture medium. Error bars indicate the standard deviations.

controls at 37°C $P < 0.05$). Laval and Michel (26) found similar results for rat hepatocytes (H4-cells), where in aerobic conditions DNP considerably decreased the ATP concentration after 5 hr at 41°C. In our previous work (9) we have shown that 42.5°C in itself has no significant effect on the nucleotides. Thus, the data indicate that ATP production from glycolysis is sufficient to prevent NAD^+ depletion from γ radiation, but not after hyperthermia because glycolysis does not proceed sufficiently to prevent ATP depletion in cells cultured in glucose-supplemented media that have ATP regeneration from oxidative phosphorylation blocked by DNP.

Adenosine, hyperthermia, and γ radiation. This experiment was done to see if exogenously supplied adenosine could potentiate ATP production after damaging the mononuclear leukocytes with hyperthermia and γ radiation. Human and calf sera contain adenosine deaminase, but horse serum is free of this enzyme (31).

TABLE II

Pool Sizes of ATP and NAD^+ Heated and Irradiated Mononuclear Leukocytes That Had Been Subsequently Incubated for 5 hr at 37°C with 100 μM Adenosine plus 1 mM Uridine

Treatment	ATP (pmole/10 ⁶ cells)	NAD^+ (pmole/10 ⁶ cells)
(A) 37°C ($t = 0$ hr)	1550 $\pm$ 130	170 $\pm$ 16
(B) 37°C ($t = 5$ hr)	2015 $\pm$ 180	220 $\pm$ 20
(C) 42.5°C ($t = 5$ hr)	1950 $\pm$ 180	224 $\pm$ 20
t test A vs B	$P < 0.1$	$P < 0.1$
t test B vs C	NS	NS
(D) 37°C + 30 Gy ($t = 5$ hr)	1800 $\pm$ 180	160 $\pm$ 15
(E) 42.5°C + 30 Gy ($t = 5$ hr)	1000 $\pm$ 100	90 $\pm$ 9
t test D vs E	$P < 0.05$	$P < 0.05$

Note. Data from three blood donors.

Therefore, we have used horse serum in this experiment to prevent exogenous breakdown of the added adenosine. High doses of adenosine are toxic to cultured cells presumably due to the imbalance in the supply of purines or pyrimidines (31–33). It has been found that 1 mM uridine abolishes the toxic effect of adenosine. Hence, when we wanted to supply the cells with exogenous adenosine as an ATP precursor, we also added 1 mM uridine to the culture medium. Figure 2 shows the ATP pool versus the concentration of adenosine after a 5-hr incubation at 37°C. The error bars indicate standard deviations of multiple measurements. The ATP level increases with increasing adenosine concentration up to about 100 μ M and then drops off slowly.

Exogenously supplied adenosine at 100 μ M was found to be optimal for increasing the ATP pools in mononuclear leukocytes. Therefore, we have chosen this adenosine dose to study the effects of hyperthermia and γ radiation on NAD⁺ and ATP pools. The results are shown in Table II. Exogenous adenosine generated a similar increase of the ATP pool 5 hr after hyperthermia as it also did after control incubation at 37°C. Gamma radiation at 37°C lowered the capacity of ATP generation from adenosine but it was still enlarged (i.e., above the control value at $t = 0$). However, the combination of hyperthermia and γ radiation interfered drastically with the phosphorylation of adenosine to ATP.

The NAD⁺ pools after hyperthermia and normothermia were identical. As in our previous work (9), the NAD⁺ pool reached a higher level after γ radiation compared to γ radiation plus hyperthermia (Table II).

Morphologic alternations following hyperthermia and γ radiation. Figures 3a–d show typical electron microscope (EM) photomicrographs of lymphocytes after various treatments and a 5-hr repair incubation at 37°C. The control lymphocytes (Fig. 3a) had normal nuclei, several free ribosomes and polysomes, sparse endoplasmic membranes with ribosomes, a few Golgi structures, and a moderate amount of mitochondria with well-preserved cristae.

The mitochondrial cristae and the endoplasmic membranes were slightly dilated in some lymphocytes treated at 42.5°C. At 37°C plus 30 Gy γ radiation (Fig. 3b) the mitochondrial cristae were also slightly dilated. The endoplasmic cisternae in some lymphocytes were heavily dilated as was the perinuclear space. The same changes, though much more pronounced, were found at 42.5°C plus 30 Gy radiation (Figs. 3c and d). Nuclear fibrillar structures were visible in a few lymphocytes (Fig. 3d). Flocculations within the mitochondria of the type described by Bowers *et al.* (34) in perfused rat liver cells were not encountered. The number of hairlike structures (microvilli) on the surface of lymphocytes decreased at treatment with 37°C as well as 42.5°C prior to 30 Gy γ radiation.

DISCUSSION

Recently, we have reported (9) on a study comparing DNA repair synthesis, cellular NAD⁺ levels, and ATP, ADP, and AMP pools in human mononuclear leukocytes following exposure to hyperthermia and γ radiation separately and in combination. Gamma-ray-induced DNA repair synthesis was inhibited by hyper-

thermic treatment indicating the possible involvement of ADPRT. This was also verified by the nucleotide data, which showed that hyperthermia and γ radiation interact synergistically by inhibiting the recovery of depleted cellular NAD^+ levels. Since NAD^+ is a substrate for ADPRT, then these data implicated a reduced ADPRT activity as a possible molecular mechanism for the radiosensitizing effects of hyperthermia.

In this paper we have measured the activity of ADPRT in human mononuclear leukocytes after treatment with various combinations of hyperthermia (42.5°C) and γ radiation (30 Gy). Gamma radiation was found to activate the enzyme considerably, but we found no further effect on the activation level (within the experimental accuracy) when the cells were exposed to hyperthermia prior to the radiation (Fig. 1).

Nolan and Kidwell (22) found for *Drosophila* nuclei that ADPRT is temperature sensitive. The optimum temperature for *Drosophila* cells was found to be 20°C , and at 37°C the ADPRT activity was lost. Compared to this large temperature span a change from 37°C at 42.5°C is very small. However, at very high temperatures (above 45°C) we found that the ADPRT activity will also be considerably reduced for mononuclear leukocytes (our laboratory, unpublished data).

In our previous paper (9) we have shown the ATP pools are reduced by moderate hyperthermia (42.5°C for 45 min) but they are restored within about 5 hr. ATP is a direct precursor to NAD^+ . Hence we reasoned that under the influence of continuous NAD^+ depletion via ADPRT stimulation by γ -ray-induced DNA strand breaks, limited supply of ATP during peak ADPRT activity may result in a more permanent reduction of NAD^+ pools. To test this hypothesis, we have uncoupled oxidative phosphorylation with $100\ \mu\text{M}$ 2,4 dinitrophenol and then examined NAD^+ pool depletions after damaging the cells with γ radiation but in the absence of any ATP regeneration (Table I). Cells given γ radiation did not have reduced NAD^+ pools unless glucose was omitted from the culture medium. DNP does not affect ATP production from glycolysis. Hence, these data together with previous data (9) suggest that ATP production from glycolysis is sufficient to prevent NAD^+ depletion from γ radiation but not after hyperthermia because Hanks' Eagle medium contains glucose yet NAD^+ was depleted (Table I).

This observation is confirmed by supplying the cells with an exogenous source of $100\ \mu\text{M}$ adenosine and in the presence of $1\ \text{mM}$ uridine to block the depletion of pyridine nucleotide pools and thus avoid cytotoxicity (31–33). Cells given hyperthermia plus γ radiation could not fully utilize the exogenously supplied ATP precursors, which also indicates a severe limitation of ATP regeneration in the presence of ATP demand (Table II).

The large decline in NAD^+ observed following treatment with heat and γ radiation is important since the level of NAD^+ then most probably will be rate limiting for ADPRT activity. The concentration of NAD^+ in undisturbed human mononuclear leukocytes is about $400\ \mu\text{M}$ and at least at the reduced levels we obtain after damage, ADPRT will not be saturated, the K_m value in permeabilized cells being about $300\ \mu\text{M}$ (13).

There are other studies reported that hyperthermia can cause a reduction of cellular ATP (35–37). However, there are also works which have not shown any

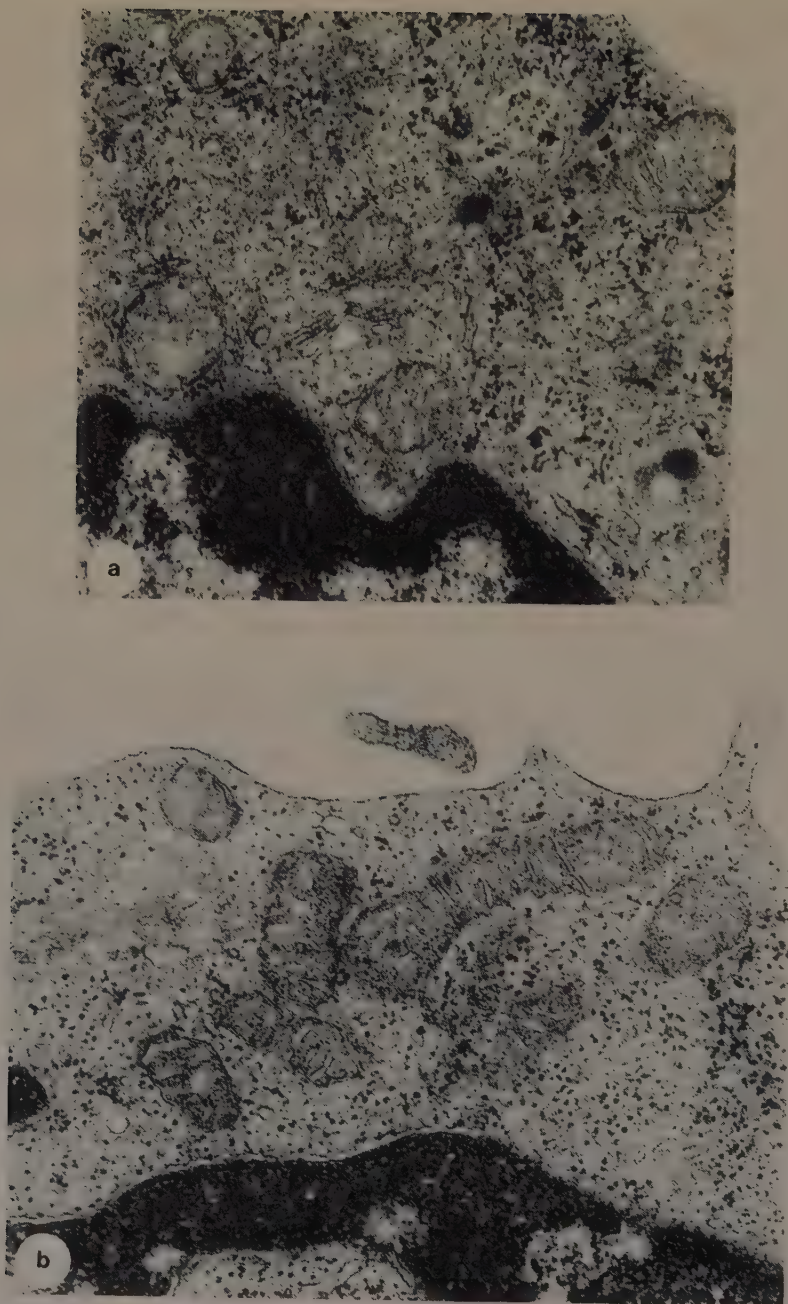


FIG. 3. Electron microscope pictures of lymphocytes 5 hr after various treatments. a, controls at 37°C ($\times 48600$); b, 30 Gy γ radiation ($\times 48600$); c, d, 42.5°C plus 30 Gy ($\times 42930$ and $\times 24750$, respectively).

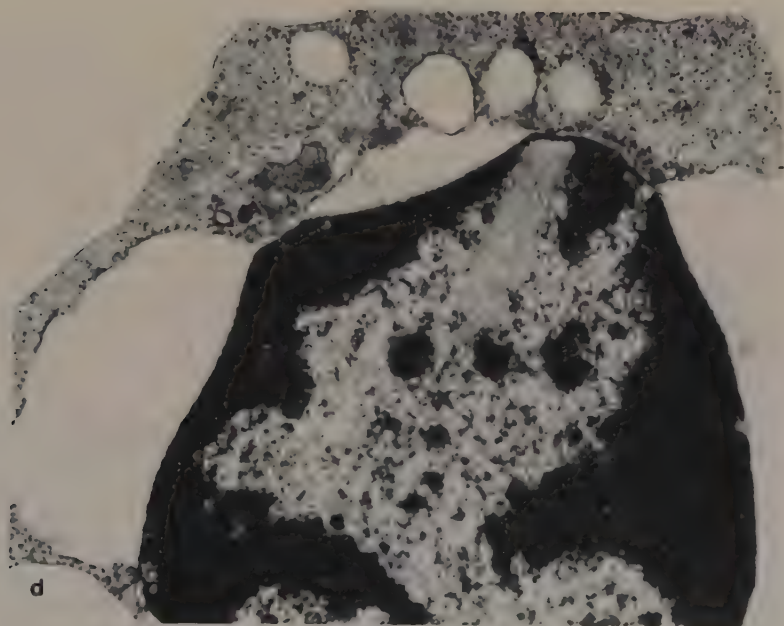
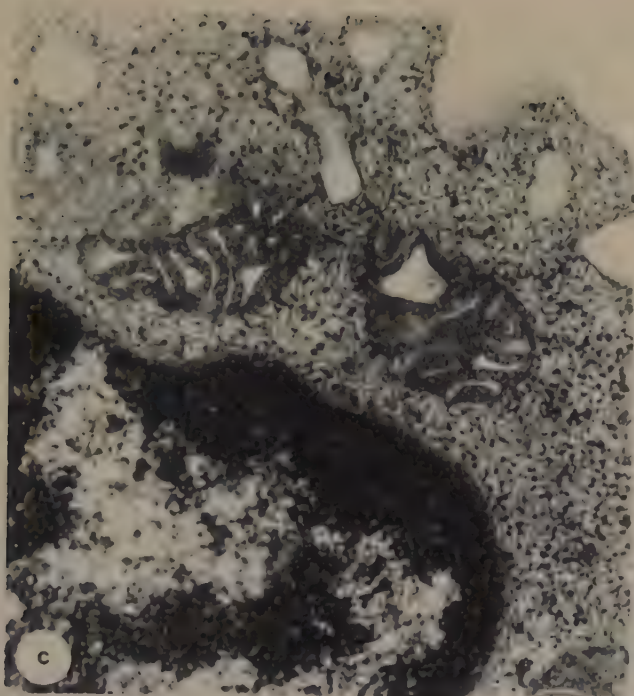


FIG. 3—Continued.

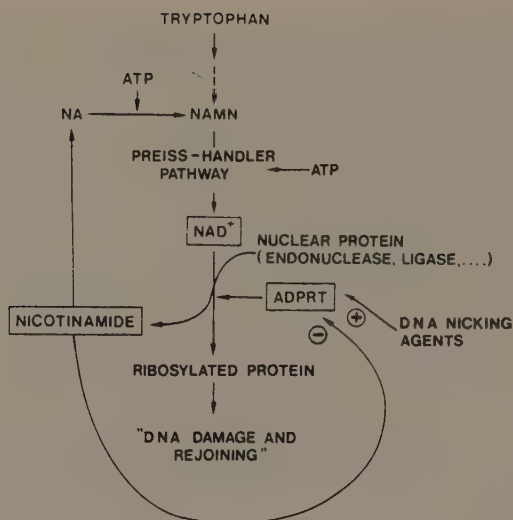


FIG. 4. Proposed mechanism for the generation and catabolization of NAD^+ in mammalian cells. Abbreviations: NA, nicotinic acid; NAMN, nicotinic acid mononucleotide.

effect of heat on ATP levels (37–39). This discrepancy could be due to the different cell types used having different sensitivity to heat which may be important when identifying target cells for hyperthermia effects.

The functional effects of hyperthermia are probably at least to some extent related to the morphologic alterations seen with electron microscopy. Hyperthermia has been found to induce reversible nucleolar lesions and clustering of perichromatin granules (40). Bowers *et al.* (34) found in hepatocytes of perfused rat liver flocculent dense bodies in the mitochondria when exposed to hyperthermia above 42°C . We find for 42.5°C plus 30 Gy γ radiation, i.e., when there is a demand for ATP to regenerate NAD^+ pools, typical dilated mitochondrial cristae (Fig. 3). This is a further indication that hyperthermia effects on mitochondria could be of considerable importance for utilization of oxygen and ATP production in the leukocytes studied.

As a hypothesis of the mechanism of hyperthermia we suggest that hyperthermia and inhibition of ADPRT act on the same level to influence DNA damage and/or rejoining; i.e., inhibitors of ADPRT prevent poly ADP-ribosylation of nuclear proteins, whereas hyperthermia affects ATP production and then the regeneration of NAD^+ which, in turn, means less substrate for poly ADP-ribosylation. Figure 4 is a summary of what is known about the synthesis and regeneration of NAD^+ for mammalian cells together with its catabolism via ADPRT (23, 41). The mechanism of rapid (re)generation of large amounts of NAD^+ for the formation of poly ADP-ribosylated proteins might be of greatest importance in regulation of DNA damage and/or repair (42). Blocking the action of ADPRT by inhibition of the enzyme or by substrate reduction (i.e., by hyperthermia) might mean accumulation of DNA damage and the cytotoxic consequences thereof. Studies of eventual additive effects

of hyperthermia plus ADPRT inhibitors when combined with γ radiation *in vitro* and *in vivo* are in progress in our laboratory.

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(Department of Pathology)

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UTILIZATION OF THE PIXE-METHOD FOR ELEMENTAL ANALYSIS IN HUMAN LYMPHOCYTES
AND ITS APPLICATION TO EFFECTS CAUSED BY HYPERTHERMIA¹

Göran G. Jonsson ^{§+2}, Anders Olsson⁺, and Ronald W. Pero⁺

[§]Department of Oncology, University Hospital, S-221 85 Lund, Sweden

⁺Department of Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund, Sweden.

Jan Pallon, Ann-Kristin Ekholm, and Sven A.E. Johansson

Department of Nuclear Physics, Lund Institute of Technology, Sölvegatan 14,
S-223 62 Lund. Sweden.

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²To whom all correspondence should be addressed **under** address: Department of Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund, Sweden.

ABSTRACT

The Particle-Induced X-ray Emission method (PIXE) is a fast and sensitive method for multi-element trace analysis. The PIXE method is described and its value for analysis of elements in biological samples is pointed out. The PIXE method and atomic absorption spectrometer are utilized for measuring intracellular levels of ions in human lymphocytes exposed to supra normal temperatures - hyperthermia. We find no significant effects of hyperthermia on protein-bound metals but observe a reduced Na-K ion pumping capacity. Possible backgrounds to this finding are discussed.

Metal ions are of essential importance in biochemistry. The intracellular ion balance is crucial for maintaining osmotic pressure and electric potential across membranes. Some ions are required for protein synthesis and for activity of numerous enzymes. Other ions play a role in the transport of amino acids and sugars. There are also many rare ions, so called trace elements, of importance for biological processes (Prasad, 1976). Both reduced and increased levels of trace elements are known to cause serious consequences for cellular life, although the action of many of them are unknown.

Knowledge about intracellular levels of ions is of great interest for the understanding of biological processes following treatments with radiation, hyperthermia, antibiotics and other drugs, as well as in ecotoxicology.

One method for measuring many elements in one single experiment is the Particle-Induced X-ray Emission method - PIXE (for reviews see Johansson and Johansson, 1976, Khan and Crumpton, 1981 or Martin, 1984). The sample is irradiated with protons in the MeV range for excitation of the atoms. The de-excitation characteristic X-rays are then measured with a solid state detector. High sensitivity makes the PIXE method a suitable tool for routine multi-element trace analysis also for biological samples.

The aim of this paper is two-fold:

- (i) Comparison of the PIXE method with the more commonly employed method of atomic absorption for analysis of biological samples;
- (ii) Utilization of the PIXE method for measuring intra-cellular levels of ions in human lymphocytes after exposure of the cells to supra normal temperatures. The PIXE data are compared with similar data obtained from the same blood donors with an atomic absorption spectrometer.

Temperatures above the normal body temperature - hyperthermia - is known to induce many biological effects (Dietzel, 1975, Dewey et al, 1977, Field and Bleehen, 1979). Important targets for hyperthermia are the cell membranes (Wallach, 1978). Hyperthermia may affect lipid composition, fluidity and permeability of the membranes for various molecules and ions (Yatvin 1977, Cress et al, 1978, Kwock et al, 1978, Yi, 1979, Yi et al, 1983a, 1983b).

We here report data obtained by the PIXE method and atomic absorption spectrometer, on intra-cellular contents of various ions following exposure of human lymphocytes to hyperthermia between 37 and 46°C. The two methods give comparable results, although the PIXE method is much more sensitive than the atomic absorption spectrometer. The data at control temperature (37°C) are comparable to those reported earlier in the literature.

At temperatures above 43°C the level of K decreases rapidly with increasing temperature with a simultaneous but smaller gain of Na. There is one earlier experiment by Yi et al (1983a) on mouse mastocytoma P 815 and HeLa cells also showing these effects. We find no significant effect of hyperthermia on protein-bound metals like Fe, Zn and Mg.

We suggest that the reduced efficiency of ion pumping observed could be related to the suppressed ATP production at high temperatures reported earlier by us for mononuclear leukocytes (Jonsson et al, 1984, 1985).

MATERIAL AND METHODS

Particle-Induced X-ray Emission (PIXE)

General

PIXE is a fast and sensitive method for determining elements in small samples (milligrams to grams) (Martin, 1984). The method can quantify elements heavier than Al simultaneously. The lowest detectable amount is in the order of parts of nanograms, while concentrations are measured at the ppm-level.

Principle

Protons (or heavier ions) with an energy of 2-3 MeV are produced by an accelerator. When the ions hit the sample characteristic X-rays are emitted. By measuring the radiation with an energy-dispersive X-ray detector - a Si(Li) detector - multielemental quantitative analysis can be performed. A routine analysis is carried out in 1-10 minutes.

PIXE is an analytical method similar to X-ray fluorescence (XRF), however, in that case the characteristic X-rays are induced by irradiation from a X-ray source. One advantage of PIXE compared to XRF is the possibility of analysing samples with very small dimensions. The proton beam can be focussed to micrometer dimensions and scanned across a sample in the same manner as with an electron microprobe. The sensitivity when using PIXE is often 1-100 times better than with electron microprobe, but the spatial resolution is not so good.

Set up

The proton beam was produced by the 3MeV Pelletron tandem accelerator at the Lund Institute of Technology. Samples were analysed in the PIXE routine chamber having an 8 mm diameter proton beam with an energy of 2.55

MeV (Fig. 1). The X-rays were measured with an Kevex 80 mm² Si(Li)-detector connected to a Nuclear Data ND6620-system and evaluated on a Norsk Data ND 100/500 computer system using the X-ray spectrum fitting/evaluating computer code HEX.

In this paper all samples analysed were prepared to be thin samples. This was done by pipetting 20 µl of the cell suspensions onto Kimfol foils (a polycarbonate film, 180 µg/cm², Kimberly Clark).

Calibration and quantification

PIXE is an absolute method relying on such physical parameters as proton energy, X-ray cross-sections, detector efficiency, solid angle, and the X-ray transmission between sample and detector, in other words parameters that can be checked or controlled. Once the set-up is calibrated

can be used for determining amounts in unknown samples without the need of running elemental standards at the same time. However, to verify the correct condition of the PIXE set-up, a Ag-plate is analysed as first sample at the beginning of each batch of samples. With this check the quality of the analysis can be guaranteed.

Quantification is carried out by relating the number of pulses in a X-ray peak to the amount of protons that have caused the emission of the radiation. The amount of protons is determined by measuring the charge with a current integrator. The accumulated charge was typically 10 µC. The number of pulses/charge unit for a certain element, can directly be converted to a value of mass by using the calibration data. The precision and accuracy of the analysis is better than 10% if the number of pulses in the determined peak is larger than 10000.

Atomic absorption spectrometer

The analysis were carried out on a Varian Techtron instrument (Varian

Inc.) at the Department of Clinical Chemistry, University Hospital in Lund. Cells were lysed with 1% LaCl_3 in glass distilled water. LaCl_3 is a complexing agent which allows the release of Ca and Mg for accurate measurements. LaCl_3 was run as blank when analysing all elements. 150 μl of the suspension of lysed cells were injected into the spectrometer. Fuel was C_2H_2 with air as oxidant. Both lysates and blanks were diluted to ion concentrations in the linear range of the spectrometer calibration curves.

Isolation of lymphocytes

All glasswares used in these experiments were washed in 35% HNO_3 and then rinsed four times in distilled water which had been purified with ultrafiltration (Milli-Q-system, Millipore AB, Sweden). Physiological saline was prepared with ultrafiltrated water and NaCl (pro analysi, Merck).

Peripheral blood was taken by venous puncture into Vacutainers without any anti-coagulant from obviously healthy blood donors. The blood was carefully transferred to a glass flask and gently swirled with glass beads to defibrinate it and remove the platelets. The defibrinated blood was centrifuged at 100 x g for 15 min and the serum removed. The blood cell fraction was diluted to original volume with physiological saline and layered on a Isopaque-Ficoll cushion (Lymphoprep, Nyegaard and Co.), which was centrifuged at 600 x g for 20 min. The mononuclear leukocytes were collected from the interphase zone and washed three times in physiological saline. The number of cells were counted in a Coulter counter (model ZB, Coulter Electronics). Cell distribution analysis showed that about 98% of the mononuclear cells were lymphocytes.

In order to study if the number of cells on the Kimfol foil would affect the yield of ions measured, different amounts of cells were taken out from the cell suspension; 20 μl samples adjusted to contain $0.5 - 2 \cdot 10^6$ cells were applied onto the foils and dried in dust-free atmosphere.

Hyperthermia treatment

Cultures containing about $5 \cdot 10^6$ cells were prepared at room temperature in 4 ml of Hank's Eagle Medium plus 1 ml autologous serum. The culture medium was supplemented with 2.5 mM L-glutamine, penicillin (50 $\mu\text{g/ml}$) and Streptomycin (50 $\mu\text{g/ml}$).

The cells were placed in water baths for 45 min at temperatures ranging from 37 up to 46°C. The temperature was measured with an accuracy of about $\pm 0.2^\circ\text{C}$. The heating period of 45 min was chosen mainly because that exposure time is used in the clinical application of hyperthermia as radiosensitizing agent in our hospital (Lindholm et al, 1982).

Immediately after the hyperthermia treatment the cell cultures were transferred to a 37°C incubator and maintained there for time periods up to 5 hrs to allow repair of damage.

After the appropriate incubation times the cells were spun down and washed three times in the physiological saline and 20 μl samples adjusted to contain about 10^6 cells were applied onto the Kimfol foils and dried in dust-free atmosphere.

RESULTS

Element analysis

A typical PIXE-spectrum of human lymphocytes is shown in Fig. 2. Effects on element yields of the number of cells on the Kimfol foils are shown in Fig. 3. Clearly there is good linearity up to about $1 \cdot 10^6$ cells/20 μ l. At higher densities there are probably X-ray absorption effects which cause the flattening out of yields seen in the figure. For Zn and Br with their apparent positive intercepts at zero density there is probably some contamination.

Table I shows a comparison between results obtained with the PIXE method and the atomic absorption spectrometer for the same blood donors (n=4). The two methods obviously give comparable results when carried out on the same leukocyte sample, although the detection limits are considerably lower with the PIXE-method. Although the values available in the literature for Fe, Zn, Na, and K in human leukocytes have varied considerably (Dennes et al, 1961, Segel et al, 1975, Bonnet et al, 1976, Ryan et al, 1980), our values for these metals are within the reported range.

It should be pointed out that there are big inter-individual variations of metal levels, expected mainly to be due to nutrition and health status.

Hyperthermia effects on intracellular ion contents

The potassium level at 37°C (Fig. 4) remained fairly constant up to 5 hrs of maintenance in culture (i.e. within the experimental accuracy). At 42.5°C there is indication of a loss of K soon after treatment but the level returns to control level within about 1 h. At 44°C the K pool remains low for the whole time period studied (i.e. about 50% of control level). The insert in Fig. 4 is the temperature dependence of the K level after 5 hrs repair incubation at 37°C. For Na (Fig. 7) we note a slight gain with in-

creasing temperature. At 46°C the K loss is about 75%, but the Na gain is only about 40% as measured 5 hrs after heat treatment. For all other ions measured; Zn (Fig. 5), Fe (Fig. 6), Mg, Ca and Cu (data not shown), we find no significant changes with temperature.

DISCUSSION

Element analysis with the PIXE method

From the results it is noted (Fig. 3) that when the density of cells applied onto the Kimfol foils is very high ($>> 5 \cdot 10^4$ cells/ μl) we get no linearity of ion yield versus cell density. This is probably due to absorption effects of the X-rays in the thick samples.

In PIXE the analyses is restricted to a thin surface layer of the sample. The targets must be either thinner than this surface layer (thin samples, $\leq 2 \text{ mg/cm}^2$) or thicker (thick samples, $\geq 15 \text{ mg/cm}^2$). To get correct quantification in the case of thick targets the sample must be homogenous. With thick targets it is also possible to analyse Na, Mg, Li, F and B with a complementary method, Particle Induced Gamma-ray Emission (PIGE).

PIXE is multielemental with the maximum sensitivity for elements with atomic number 25-30, see Fig. 8.

However, due to the sample composition this sensitivity can not always be achieved:

1. Large amounts of an element causes the background to be higher in the vicinity of a peak; a special case is when the K_{β} X-ray peak of an element coincides with the K_{α} peak of another heavier element (Fe on Co, K on Ca, etc);
2. Large amounts of lighter elements give rise to a more intense X-ray production and thus a high count-rate at the analysis. Due to limitations in the detection system the count-rate must be kept below

certain values. This is done by putting X-ray absorbers in front of the detector, but at the same time the sensitivity for lighter elements is reduced;

3. Some elements with high concentrations in biological matrices, e.g. Na, give rise to a general increase in the background due to the emission of γ -rays which are scattered in the X-ray detector. The reduction in sensitivity is most significant for elements heavier than As. The problem is most critical when analysing thick samples.

Hyperthermia effects on intracellular ion contents

We have found that the levels of K and Na remain relatively constant up to 43°C, above which the content of K decreases rapidly with increasing temperature (Fig. 4) with a simultaneous but smaller gain of Na (Fig. 7). Similar net changes of K and Na levels were observed by Yi (1979) and Yi et al (1983a). The levels of protein-bound elements Zn, Mg, Fe and Cu were not found to change with increasing temperature. Also the level of Ca as measured by atomic absorption spectrometer remained constant within the experimental accuracy.

The different ion currents are not expected to be independent of each other because of electric charge conservation.

Because of high background levels from the physiological saline we were not able to measure the level of Cl, but from the work of Yi et al (1983a) one can expect that the K ion current is greater than the Cl ion current which means a very important influx of hydrogen ions. This influx of H^+ means lowering of intracellular pH which for a tumor tissue with its already high acidity could be lethal.

The mechanism behind the changes of K and Na contents could be due to less effective energy-dependent ion pumping. Hyperthermia also alters amino acid transport kinetics in a way suggesting that hyperthermia either reduces

the number of carriers in the membrane or slows down the rate of transport across the membrane (Kwack et al, 1978). Furthermore $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ activity has been found to be temperature sensitive (Wisnieski et al, 1974), which in turn could affect ion pumping.

We have in an earlier paper found evidence that ATP production is suppressed in human mononuclear leukocytes exposed to hyperthermia above 43°C (Jonsson et al, 1984, 1985). This decrease of ATP pools was found to be parallel to a reduced viability as measured by trypan blue exclusion. This phenomenon has also been observed by other authors for various cell types (Francesconi and Mager, 1979, Ohyama and Yamada, 1980, Lunec and Cresswell, 1983). Reduced ATP pools is expected to have great consequences for cellular life. Especially when there is a demand of energy, which is the case after DNA has been damaged, the energy production might be critical. Reduced ATP production is expected to affect energy-dependent transport mechanisms, which in turn might limit uptake of DNA repair precursor substances and disturb ion pumping. However, hyperthermic reduction of ATP is probably not occurring in all cell lines. Yi (1979) found no change of intracellular ATP pools in the mouse mastocytoma cells exposed to hyperthermia. Similarly, Henle et al (1984) found no significant reduction of cellular ATP in HeLa cells exposed to heat. Also Gerweck et al (1984) found no immediate decrease in ATP following moderate heating under normal conditions.

There are many possible targets for hyperthermia, e.g. chromosomal damage (Dewey et al, 1978), inhibition of cellular respiration and metabolism (Cavaliere et al, 1967, Mondovi et al, 1969, Muckle and Dickson, 1971, Dickson and Shah, 1972). The cell membrane is certainly also a crucial target for heat damage, where lipid composition, fluidity and permeability of molecules and ions may be affected (Yatvin, 1977, Wallach, 1978, Cress et al, 1978, Kwack et al, 1978, Yi, 1979, Yi et al, 1983a, 1983b). Changes in intracellular ion content can alter not only the intracellular pH, critical for survival, but

also the activity of DNA repair enzymes (Spiro et al, 1982, 1983) and probably cellular proliferation capacity (Quastel and Kaplan, 1970, Segel et al, 1975). The present study supports the idea that the plasma membrane plays a significant role in the response of cultured cells to hyperthermia.

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TABLE CAPTION

Table I. Ion concentrations in human lymphocytes as measured by the PIXE method and by atomic absorption. Data obtained from the same blood donors (n=4).

TABLE I.

Ion	PIXE method		Atomic abs. spectrometer	
	Content of ion (fmol/cell)	Detection limit (fmol/cell)	Content of ion (fmol/cell)	Detection limit (fmol/cell)
Na	N.D.*	-	$\approx 3^{\pm 1}$	1 ?
Mg	N.D.	-	$0.8^{\pm 0.1}$	0.2
K	$5.9^{\pm 0.6}$	0.2	$10^{\pm 2}$	2
Ca	N.D.	-	$1.3^{\pm 0.4}$	0.5
Fe	$0.8^{\pm 0.1}$	0.01	N.D.	1 ?
Cu	$0.02^{\pm 0.01}$	0.005	$0.03^{\pm 0.02}$	0.02
Zn	$0.03^{\pm 0.01}$	0.005	$0.05^{\pm 0.02}$	0.02
Br	$0.013^{\pm 0.004}$	0.005	N.D.	-

* N.D. = Not Determined

FIGURE CAPTIONS

- Fig. 1. Schematic drawing of a PIXE analysing/evaluating system.
- Fig. 2. Typical PIXE-spectrum for human lymphocytes. Cell density 0.5×10^6 cells/20 μ l physiological saline.
- Fig. 3. Intracellular ion levels of K, Fe, Zn and Br versus cell density on the Kimfol foils. Error bars show the standard deviations.
- Fig. 4. Intracellular level of K versus incubation time at 37°C following exposure of the lymphocytes to 37, 42.5 and 44°C for 45 min. Inserted figure shows the K level versus temperature 5 hrs after heat exposure. Significance levels of deviation from contravalue at 37°C are indicated. The cell density was 1.0×10^6 cells/20 μ l physiological saline.
- Fig. 5. Intracellular level of Zn versus incubation time at 37°C. See figure caption to Fig. 4.
- Fig. 6. Intracellular level of Fe versus incubation time at 37°C. See figure caption to Fig. 4.
- Fig. 7. Intracellular level of Na versus temperature 5 hrs after heat exposure. The analysis was done with atomic absorption spectrometer.
- Fig. 8. The detection limits in ng/cm^2 for consecutive elements with PIXE at a routine analysis of a backing Kimfol foil.

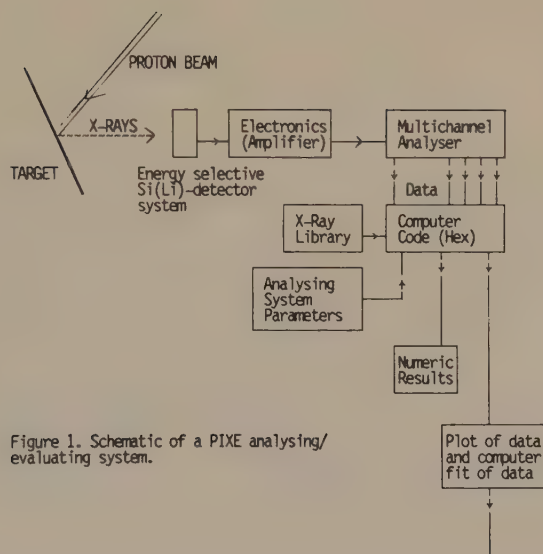


Figure 1. Schematic of a PIXE analysing/evaluating system.

Figure 1

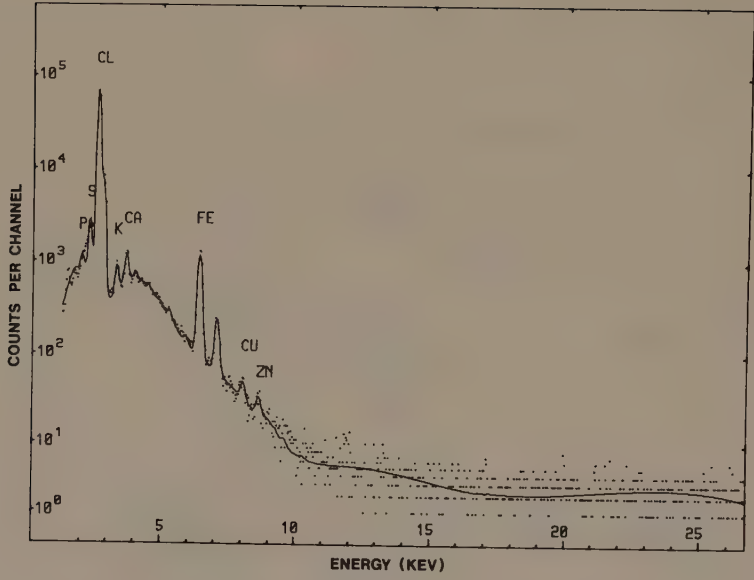


Figure 2

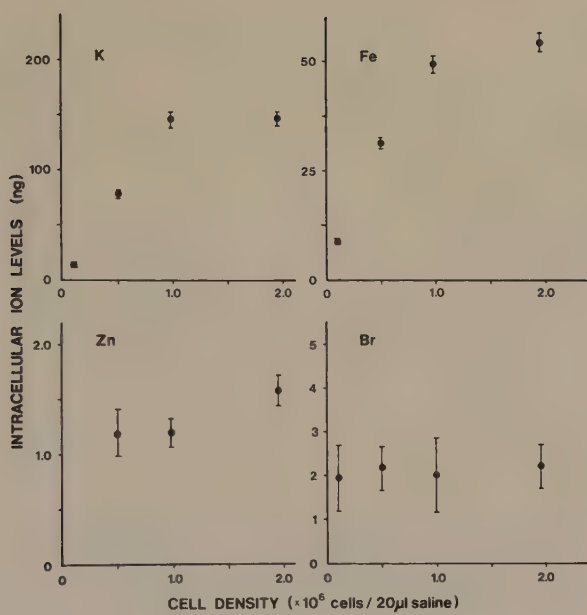


Figure 3

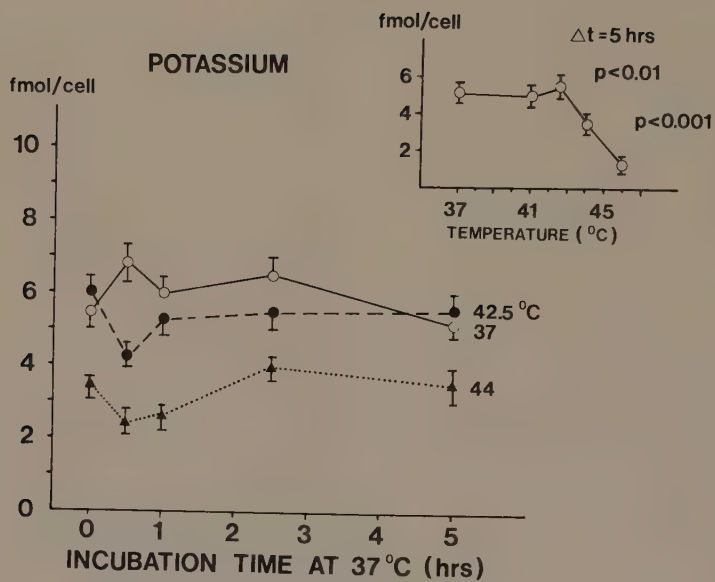


Figure 4

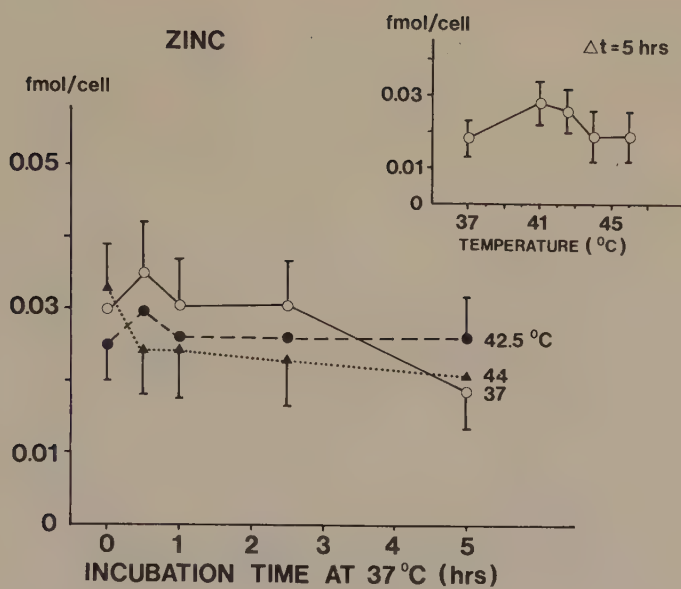


Figure 5

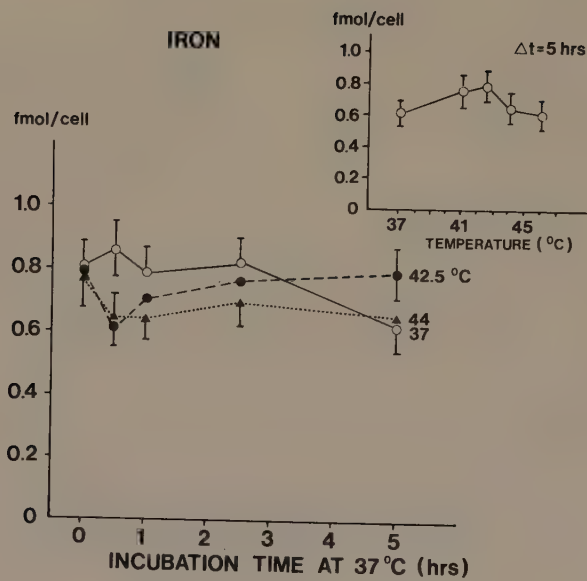


Figure 6

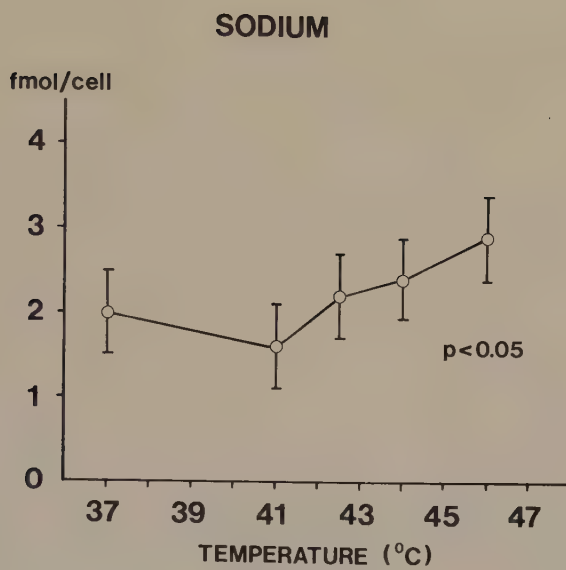


Figure 7

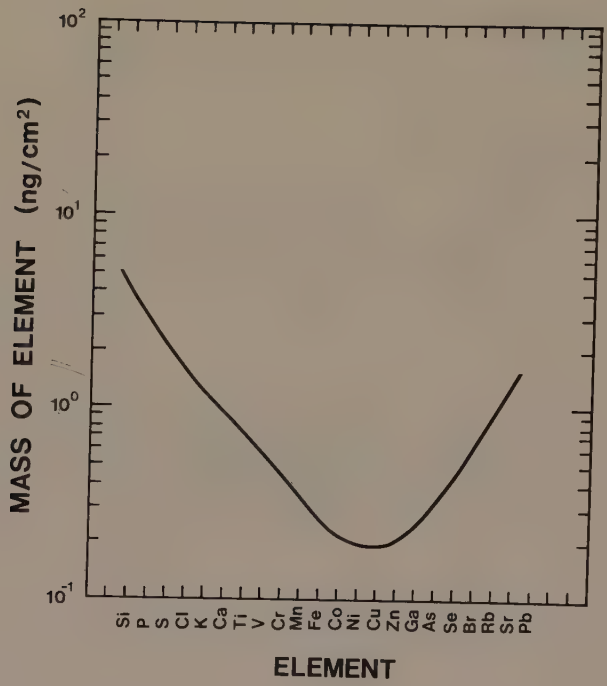


Figure 8

Nicotinamide as a radiosensitizer of a C3H mouse mammary adenocarcinoma

Göran G. Jonsson^{1,2}, Elisabeth Kjellén¹ and Ronald W. Pero²

Departments of ¹Oncology and ²Biochemical and Genetic Ecotoxicology, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07, Lund, Sweden

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Key words: Nicotinamide; Radiosensitization

Summary

Inhibitors of the chromosomal enzyme ADP-ribosyl transferase, like nicotinamide, have been shown to inhibit DNA strand rejoining and also to potentiate the lethality of DNA damaging agents in vitro. We have examined the radiosensitizing potential of nicotinamide in vivo by using transplanted C3H mouse mammary adenocarcinomas as our model system. Our data indicate a radiosensitizing effect for nicotinamide.

Introduction

Nicotinamide, known as vitamin B₃, has been found to be a potent inhibitor of the chromosomal enzyme ADP-ribosyl transferase (ADPRT) [9,16]. This enzyme catalyses the polymerization of ADP-ribose moieties derived from NAD⁺ to chromatin proteins [9]. ADPRT is activated by genotoxic agents [2,7,10,15] and thereby the NAD⁺ pools become depleted [12,17,20]. It has been suggested that ADPRT has a function in the control of breaking and rejoining DNA. There are at least two different hypotheses concerning the function of the enzyme in DNA repair, namely, control of endonuclease activity [1,3,19] and/or ligase activity [6] by ADP-ribosylation. Inhibitors of the enzyme have been found to retard DNA strand rejoining and they, likewise, potentiate the lethality of DNA damaging agents in vitro [7].

The apparent role of nicotinamide as an inhibitor of ADPRT and its function as a potentiator of lethality by DNA damaging agents like γ -radiation, has stimulated us to investigate further eventual effects of nicotinamide as a radiosensitizer in vivo utilizing C3H mouse mammary carcinoma as a model system. There has been a previous report that new NAD⁺ synthesis induced by large doses of nicotinamide radiosensitizes sarcoma S.180 tumors [5].

Materials and methods

Mammary carcinoma transplantations

The tumors were transplants derived from a single spontaneous mammary adenocarcinoma occurring in a C3H mouse (kindly given to us from Karolin-

ska Institutet, Stockholm). Viable pieces of the original tumor were taken out and pressed through a Borell strainer into Parker tissue culture medium (Gibco). The extract was centrifuged at 400 g for 5 min and the supernatant decanted. The pellet was suspended in 10 ml culture medium and an aliquot was taken out for cell counting. The cell suspension was then adjusted to about 2×10^6 cells/ml culture medium. An 0.15 ml aliquot of this cell suspension was injected subcutaneously above the right gastrocnemius muscle of about 2 months old C3H mice who had an average weight of 24 g. The tumor take was routinely close to 100%. Seven days after inoculation the tumors had grown to an average diameter of about 8 mm which was a suitable size for easy palpation and estimation of tumor volume.

Treatment with nicotinamide

Nicotinamide at about a concentration of 2 mM has been found to be optimum to inhibit ADPRT in cultured cells treated with genotoxic agents [3]. Hence, this concentration of 2 mM nicotinamide, corresponding to about 0.2 g nicotinamide/kg mouse, was chosen as a radiosensitizing dose. This dose of nicotinamide is far below the $LD_{50} = 1.68$ g/kg found for rats when administered intraperitoneally [13]. A nicotinamide dose of 1.68 g/kg given to C3H mice was found not to be lethal for the animals.

Irradiation procedure

The C3H mice with transplanted tumors of the appropriate size (i.e. about 8 mm diameter) were given radiotherapy without and with nicotinamide injected intraperitoneally. Radiation was given with a ^{60}Co γ -ray source (Siemens, gammatron S) that had a dose rate of 1.4 Gy/min. A water bolus, 5 mm thick, was used to get a homogenous dose in the tumor. Shielding of the animal was accomplished with a 80 mm thick lead block which had a circular aperture of 15 mm diameter. Non-anesthetized animals were placed in a plexiglas holder so that the right leg could be stretched out and the tumor carefully adjusted into the radiation field of the aperture.

Estimation of tumor volume

The tumors were regarded as prolate spheroids with minor and major semi-axes, a and b , respectively measured in the sagittal plane of the animal. The tumor volume (V) was then calculated by the formula

$$V = \pi a^2 b / 6.$$

Results and discussion

In a separate experiment we have found (data not shown) that ^{14}C -labeled nicotinamide given intraperitoneally is very rapidly distributed within the mice. Thus a 30-min period was chosen as a suitable time between intraperitoneal administration of nicotinamide to the mouse and the irradiation. It has been found previously that the absolute levels of nicotinamide and its metabolites remain almost constant over long time periods even at a concentration as high as 2 mM [11]. In rat liver the half-life of nicotinamide has been found to be 4–8 days [18]. However, it should be noted that peak tumor concentration of radiosensitizer may not necessarily coincide with peak radiosensitization for a hydrophilic compound like nicotinamide [4].

Tumor growth curves are shown in Fig. 1. The

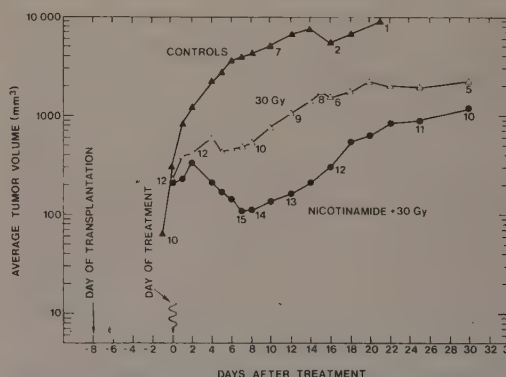


Fig. 1. Average tumor volume versus time after the various treatments. The numbers appearing next to the data points indicate how many animals were living at that time.

TABLE I

The effect of nicotinamide and γ -radiation on the growth of mammary carcinomas transplanted to the right leg of C3H mice.

Days after treatment	Total no. of living animals ^a	Living animals with tumor remission ^a	Living animals with tumor relapses ^a	Average tumor volume $\bar{V} \pm \text{S.E.M. (mm}^3\text{)}$ ^a
A. Untreated controls (10 animals from beginning)				
7	10	0	—	3856 $\pm$ 546
10	7	0	—	5088 $\pm$ 1031
15	7	0	—	7690 $\pm$ 1128
20	1	0	—	8418
25	0	—	—	
30	0	—	—	
B. 30 Gy (12 animals from beginning)				
7	12	1	—	475 $\pm$ 219
10	10	3	—	778 $\pm$ 417
15	8	3	—	1798 $\pm$ 1181
20	6	0	3	2268 $\pm$ 2065
25	6	0	3	1913 $\pm$ 1620
30	5	0	3	2204 $\pm$ 1950
C. Nicotinamide plus 30 Gy (15 animals from beginning)				
7	15	9	—	111 $\pm$ 45
10	14	9	—	134 $\pm$ 67
15	13	8	2 ^b	240 $\pm$ 128
20	12	6	3	627 $\pm$ 390
25	11	6	2	913 $\pm$ 498
30	10	6	1	1322 $\pm$ 831

^a The significance levels of differences in average tumor volume in the time region 6–20 days (all data points in Fig. 2) are A vs. B = $p < 0.001$ and B vs. C = $p < 0.01$ determined by a generalized t -test. The significance levels of differences in B vs. C on tumor remission is $p < 0.05$, animal survival $p < 0.2$, tumor relapses $p < 0.2$, respectively determined by Mantel Haenzel's test [14] and Chi-square test.

^b These two animals got tumor relapses well outside the radiated area of primary tumor, and therefore, these tumor sites were probably not properly irradiated.

more important tumor growth delay when nicotinamide is administered 30 min before irradiation is easily seen in the figure. Tumors in mice given nicotinamide only did not differ in growth rates from the untreated control tumors (data not shown). The significance levels of differences for the time period 6–20 days after treatment between controls and γ -ray treated tumors, and between tumors in animals given-only γ -radiation and those who got a combined treatment of nicotinamide plus γ -radiation, were $p < 0.001$ and $p < 0.01$, respectively (two-tailed

generalized Student's t -test). Untreated mice were all dead 4 weeks after inoculation.

A different analysis of the results is given in Table I, which shows average tumor volumes, cases of tumor remission, and relapses after various time periods for the different treatments. Utilizing a log rank test (Mantel Haenzel's test) [14] for statistical comparison, the combined treatment of nicotinamide plus radiation gave a higher incidence of tumor remission than radiation treatment alone with $p < 0.05$ (two-tailed test). There was also a consist-

ent trend toward better survival (however, non-significant $p = 0.17$) and fewer relapses among the mice given a combined nicotinamide-radiation treatment than after radiation treatment alone ($p = 0.18$) (Chi-square test with Yates' corrections).

The mechanism by which nicotinamide acts as a radiosensitizer is not known. However, it is well documented that nicotinamide can serve as a direct precursor for NAD^+ synthesis [8] as well as act as an inhibitor of ADPRT [9,16]. The possibility of a role for NAD^+ in radiosensitization is suggested by the fact that radioprotective agents such as cysteamine, 2-amino-ethylisothiuronium bromide and serotonin cause a rapid fall in tissue NAD^+ levels [5]. However, DNA-damage itself causes a severe depletion in the cellular levels of NAD^+ [12,17,20] which makes a clear interpretation of radioprotection by this mechanism difficult to understand. On the other hand, inhibitors of ADPRT such as nicotinamide and 3-amino-benzamide enhance the cytotoxicity induced by γ -radiation [15] or alkylating agents [7,15]. There is no solution to this apparent discrepancy at present, and the answer will have to await further experimentation. Nonetheless, nicotinamide is essentially free from side effects and very high doses have been tolerated by humans [21]. These facts make further studies of nicotinamide as a radiosensitizer especially attractive for future development as a sensitizing agent in the radiotherapy of human cancers.

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RADIOSENSITIZATION EFFECTS OF NICOTINAMIDE ON MALIGNANT AND NORMAL TISSUE¹

Göran G. Jonsson², Elisabeth Kjellén, Ronald W. Pero, and Robert Cameron

Department of Oncology, University Hospital, S-221 85 Lund, Sweden (G.G.J., E.K.)
Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Box 7031,
S-220 07 Lund, Sweden (G.G.J., E.K., R.W.P.)

Department of Pathology, University Hospital, S-221 85 Lund, Sweden (R.C.)

Running title: Radiosensitizing effects of nicotinamide

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²to whom correspondence should be addressed. Molecular Ecogenetics, Wallenberg Laboratory, University of Lund, Box 7031, S-220 07 Lund, Sweden.

ABSTRACT

Inhibitors of the chromatin associated enzyme adenosine diphosphate ribosyl transferase (ADPRT) have been found to inhibit DNA strand rejoining and to potentiate lethality of DNA damaging agents both in vivo and in vitro. We have in this work examined the radiosensitizing potential of one such inhibitor - nicotinamide - on tumor tissue by using transplanted C3H mouse mammary adenocarcinomas and on normal tissue in a tail stunting experiment using BALB/CA mice. Our data indicate a radiosensitizing effect of nicotinamide on tumor cells as well as on normal tissue. The data indicate a possible role of ADPRT inhibitors as a sensitizing agent in the radiotherapy of malignant tumors.

INTRODUCTION

Adenosine diphosphate ribosyl transferase (ADPRT) is a chromatin associated enzyme which covalently attaches ADP-ribose moieties derived from NAD^+ to chromatin proteins (1,2). The enzyme is dependent on DNA and histones for activity and is strongly stimulated by DNA strand breaks (3-5). Although the role of ADPRT in cells is not fully understood, there have been convincing data reported of its involvement in DNA repair (6-9), cell growth regulation (10,11), cellular differentiation (12-14) and gene expression (15).

Several hypotheses concerning the role of ADPRT in DNA repair have been advanced; for example, directly via ribosylation of repair enzymes or indirectly by the exertion of a regulatory function in repair processes. It has been reported (16) that endonuclease is ribosylated with concomitant inhibition of enzyme activity. Ligase has also been reported to be one acceptor protein dependent on direct ADP-ribosylation for activity (7). Furthermore, poly ADP-ribose has been reported to counteract inhibition of ligase activity by histones, thus facilitating rejoining of DNA strand breaks (17). However, there are some recent publications (18,19), where evidence is given that ADPRT activity might not be directly needed for DNA repair replication.

The polymer, which in fact has been found to have a branched structure (20), consists of ADP-ribose units coupled together in 1'→2' ribose-ribose glycosidic linkages and pyrophosphate bounds. Because of the size and highly negative charge of the polymer, poly ADP-ribosylation of nuclear proteins might cause distortions in the chromatin and facilitate the repair processes by making the DNA more accessible to repair enzymes. Since ADPRT obviously plays a role in many fundamental events, it has been suggested that activation of the enzyme is part of a general response to environmental stress (21).

The role of ADPRT in regulating DNA damage and/or repair is also supported by the fact that inhibitors of the enzyme have been found to retard DNA strand

rejoining and potentiate the cytotoxicity of DNA damaging agents in vitro (8,22-27) and also in vivo (26,28).

Nicotinamide (vitamin B₃) is known to be a potent inhibitor of ADPRT (29,30). Nicotinamide is essentially free of side effects and very high doses have been tolerated by humans (31). Thus, nicotinamide seems quite attractive to test as a radiosensitizer primarily in animal systems.

In a previous work (32) we have found that nicotinamide has a radiosensitizing effect in a system using transplanted C3H mouse mammary adenocarcinoma. Calcutt et al (28) found a radiosensitizing effect of large doses of nicotinamide on sarcoma S180 tumors.

We have now extended our work by studying the effects of various single doses of γ -radiation with and without nicotinamide on tumor tissues as well as on normal tissue in a tail stunting experiment. Furthermore in the tumor system histopathological analyses were performed including determination of mitotic indices comparing the effects of the combined treatment with γ -radiation alone.

MATERIAL AND METHODS

Tumor transplantations. The model system used was C3H mice with transplanted tumors from a single spontaneous mammary adenocarcinoma occurring in this strain of animal. The tumor had been successively retransplanted in 30 generations before our experiments and was the kind gift from the Department of Tumor Biology at Karolinska Institute in Stockholm. Viable pieces of tumor were selected and pressed through a Borell strainer into Parker tissue medium (GIBCO). After washing in medium the cell suspension was adjusted to about 2×10^6 cells/ml and a 0.15 ml aliquot injected subcutaneously above the right gastrocnemius muscle of adult C3H mice, about 2 months old with an average weight of about 20 g. Tumor take was close to 100%. The outgrown tumors were usually palpable after 5 days. The tumors were regarded as prolate spheroids with perpendicular minor and major axis a and b respectively measured in the sagittal plane of the animal. The tumor volume (V) is then given by $V = \frac{a^2 b}{2}$. About one week after transplantation the tumors had grown to an appropriate size ($\varnothing \approx 8$ mm) enabling palpation and accurate measurements. The animals were then divided into groups, with at least 10 animals in each, where tumor sizes were as comparable as possible.

Treatment with nicotinamide. Nicotinamide at a concentration of 2 mM has been found to be optimum to inhibit ADPRT in cultured cells treated with genotoxic agents (33). This concentration corresponds to about 0.2 g nicotinamide per kg mouse, which is well below the $LD_{50} = 1.68$ g/kg found for rats (34). This LD_{50} -dose was found not to be toxic for the C3H mice. On the other hand, higher doses of nicotinamide (i.e. 20 mM) were needed to radiosensitize CHO cells (35). Since doses to animals in this range are well above the LD_{50} -value, we have decided to use the practical and more safe dose of 0.2 g/kg (i.e. 2 mM).

In order to determine the time necessary for the nicotinamide to reach

equilibrium level within the tumor, ^{14}C -labeled nicotinamide (50 mCi/mmol, Amersham) diluted with unlabeled nicotinamide was injected intraperitoneally at a total dose of 0.2 g/kg. C3H mice with 7 day old transplanted mammary carcinomas were used for this study. The mice were sacrificed at various time periods after injection of nicotinamide and pieces of tumor from the periphery and center of the tumor respectively, as well as samples from the tail and liver were dissected out. These tissues were dissolved in 1 ml Soluene-350 (Packard Instr. Co) at 50°C for 1 hr after which 10 ml scintillator cocktail (Packard Instr. Co) was added, and then the radioactivity counted. The results were recorded as cpm ^{14}C -nicotinamide per gram wet tissue.

Radiation treatment of C3H tumors. Radiotherapy was given with a ^{60}Co γ -ray source (Siemens, Gammatron) with a dose rate of 0.9 Gy/min. To get a homogeneous dose within the tumor a 5 mm thick water bolus was used. The animals were carefully shielded with a 80 mm lead block which had a circular aperture of 15 mm diameter. The animals were placed, non-anaesthetized, in a plexiglass holder with the right leg stretched out and fixed in the radiation field of the aperture.

Histologic examination. C3H mice with 7 day old transplanted mammary adenocarcinomas ($\phi = 8$ mm) were treated with gamma-radiation at various doses alone or in combination with nicotinamide given intraperitoneally 30 min prior to irradiation of the tumor area. After various time periods the animals were sacrificed and pieces of tumor were dissected out and fixed in formalin. Routinely processed fragments of tumor were sectioned at 4-5 μ and stained with hematoxylin/eosin. Slides were studied with respect to tumor viability which was assessed by evaluation of the proportion of cells which were necrotic or which showed signs of degeneration (shrunken, pyknotic nuclei and dense dark

cytoplasm) (36). In addition, mitoses were counted in 10 high-power (500 x) fields and recorded as the average number of mitoses per field.

Tail stunting experiment. For this experiment 8 day old male BALB mice were used. The average weight of the mice was about 9 g. During treatment, the unanaesthetized mice were fixed in a holder well protected for radiation with the tails stretched out into the radiation field. 10 animals could be treated simultaneously. Each experimental group consisted of 10-12 animals, carefully selected so the tails were of the same length (about 45 mm) at the beginning of the experiment. Nicotinamide was given intraperitoneally 30 min before irradiation at a dose of 0.2 g/kg. The tail lengths were then measured until the tails stopped growing after about 45 days.

Statistical methods. Average tumor volumes and mitotic frequencies following the various treatments were compared with the aid of Student's t-test. Remissions and relapses were compared with significance levels calculated with Mantel Haenzel's test (37). This test combines information from all observation times.

RESULTS

The ^{14}C -nicotinamide activity in various tissues of C3H mice is shown in Chart 1 as a function of time after injection. Nicotinamide is the main precursor to NAD^+ and metabolizes further into NADP^+ and N-methyl nicotinamide (38-43). The absolute levels of these compounds have, in cultured rat kidney, been found to remain almost constant over long time periods even in the presence of nicotinamide at a concentration as high as 2 mM (42). In rat liver the half-life of nicotinamide has been found to be 4-8 days (43). It can be seen that ^{14}C -nicotinamide is rapidly distributed into the liver and the tumor (Chart 1), but more slowly into less vascularised structures such as the tail. An almost necrotic piece of tumor also shows low radioactivity. With the exception of the liver, the radioactivity seems uniformly distributed within the animal after only a few minutes when judged by comparison with the ^{14}C -nicotinamide dose originally administered to the mouse (i.e. 1.8×10^5 cpm ^{14}C -nicotinamide/g body weight). Based on the data in Chart 1 a 30 min preincubation period was chosen as a suitable time between intraperitoneal administration of nicotinamide and radiation treatment. The rapid increase in vitamin activity throughout the tissues seen in Chart 1, however, reflects not only cellular uptake but also distribution of nicotinamide in interstitial spaces. Since uptake of vitamin continues for at least one hour in cultured cells, then nicotinamide uptake may continue even after 30 min from nicotinamide supplied via the interstitial spaces (44).

Tumor growth measurements. Chart 2 shows the tumor growth for unirradiated controls and for tumors treated with various doses of γ -radiation with and without nicotinamide (0.2 g/kg) administered before the irradiation. Tumors in mice given nicotinamide alone did not differ in growth rates from those of untreated controls (data not shown). The growth pattern of untreated con-

trols is very rapid, almost exponential at first but then slows progressively with time, reflecting a decreasing growth fraction or increasing cell loss. Tumors given radiation at high doses first shrink temporarily and then continue to growth. When nicotinamide was administered prior to radiation there is, for high doses of radiation, even more pronounced shrinkage and a clear delay of regrowth. Table 1 shows average tumor volumes, cases of tumor remission and relapses 7, 14, and 28 days after treatment for the animal groups studied. Obviously there is significant tumor growth retardation (Student's t-test) when nicotinamide was given before radiation at the highest γ -ray doses (20 and 30 Gy) when compared to radiation alone. For 30 Gy the data in the time period 7-28 days after treatment show a better tumor remission rate following the combined treatment than with radiation alone, although the difference is not significant ($p < 0.1$ for a two-tailed test, as determined by a Mantel Haenzel's test (37)). However, in our previous paper (32) the combined treatment of nicotinamide plus radiation gave significantly better tumor remission than radiation alone (30 Gy) ($p < 0.05$ two-tailed test). Contrary to our previous data there is, in the present experiment, no trend towards better survival in animals given the combined treatment. Chart 3 shows the average tumor volume at day 30 after treatment versus dose of γ -radiation with and without nicotinamide given prior to the radiation. The Chart shows that there is a systematic retardation effect of γ -radiation alone on tumor growth. The ratio of the tumor volume in animals given radiation alone to the tumor volume in animals given the combined treatment of nicotinamide plus γ -radiation measured on day 30 after treatment is given in Table 2. Once again we see the significant enhancement of tumor retardation after the combined treatment, especially at the highest γ -radiation dose. From the data in Table 1 it is possible to estimate a nicotinamide-induced enhancement ratio based on isoeffect of tumor remission. Using a Poisson distribution for tumor remission probability fitted to the experimental data

by linear regression (Overgaard, personal communication) we find for 50% tumor remission on day 30 after treatment a dose of 42 Gy for radiation alone and 29 Gy for the combined treatment of nicotinamide plus γ -radiation which, in turn, gives an enhancement ratio of about 1.2 (i.e. $42 \text{ Gy}/29 \text{ Gy} = 1.2$).

Histologic examination. Histologic examination could not detect any significant difference with respect to morphologic viability (36) between animals treated with nicotinamide and those not so treated. All fragments studied showed numerous small and occasional larger coalescent areas of necrosis, typical for highly malignant tumors. The frequency of mitoses differed markedly between the two groups however. As shown in Chart 4 for 10 Gy γ -radiation the mitotic activity dropped sharply after radiation in all tumors. In those not pre-treated with nicotinamide, the number of mitoses had returned to pre-radiation levels after 12 hours but, in pre-treated animals, mitotic activity remained depressed for the entire period of observation.

After much longer time one could expect the difference to disappear as we see regrowth of tumor a couple of weeks after the combined treatment (Chart 2). At the higher γ -radiation doses the frequency of mitoses was so low that differences between the two treatments could not be determined with any accuracy (data not shown).

Normal tissue and tail stunting. Concerning the skin reaction around the tumor area following the combined treatment, we found more damage than with radiation alone, although there was good healing in almost all cases with at most a scar left at the tumor site. To get a better measure of the reaction of normal tissue we performed a tail stunting experiment using BALB mice. Chart 5 shows growth pattern of mice tails versus time after treatment with and without nicotinamide given prior to the different radiation doses. The

tail lengths at the day of treatment were about 45 mm and they grew to their final lengths within about 40 days. Retardation effects on tail growth can be seen as soon as 10 days after radiation treatment. The per cent tail stunting versus dose of radiation with and without nicotinamide given prior to radiation, is given in Chart 6. As for tumor volumes (Charts 2 and 3), γ -radiation alone induces an increased retardation effect with increasing dose. We note the significant shortening of tails following the combined treatment as compared with γ -radiation alone. From the Chart one can estimate the nicotinamide-induced enhancement ratio for isoeffect of 20% to be about 1.5 (i.e. $30 \text{ Gy}/20 \text{ Gy} = 1.5$).

DISCUSSION

The collected data indicate a radiosensitizing effect of nicotinamide on tumor cells as well as on normal tissue. The nicotinamide-induced enhancement ratio based on tumor remission on day 30 after treatment was found to be 1.2 which is comparable to radiosensitization by metronidazole in the same animal model system (45). However, due to the short observation periods our data are not directly comparable to TCD₅₀ values, which are the doses at which 50% of the tumors are locally controlled e.g. cured animal. The nicotinamide-induced enhancement ratio for isoeffect of tail stunting is of the same size as thermal enhancement ratios (TER) found for the growth of irradiated rat tails (46,47). Nicotinamide by itself gave little effect on the tail growth pattern, although animals given nicotinamide increase somewhat more in weight than the untreated controls (data not shown) and they also on the average get somewhat (but not significantly) longer tails.

Thus nicotinamide does not damage normal tissue unless the tissue is given radiation, which it usually is not unless it be in the immediate vicinity of an irradiated tumor.

It is to be expected that the mitotic activity should drop immediately after radiation damage and it is not surprising that a highly malignant tumor like the C3H mouse mammary adenocarcinoma recovers from a low γ -ray dose (~ 10 Gy) relatively quickly (Chart 4). Following the combined treatment we find a significant inhibition of recovery of cell division, which probably is the background to the retardation effects seen on the growth pattern for tumors as well as on tails (Charts 3 and 6).

The mechanism behind the radiosensitizing effect of nicotinamide is not clear. Nicotinamide is both a direct precursor for NAD⁺ synthesis (38) and also an inhibitor to ADPRT (1,29,30). Since ADPRT obviously has a role in the activity of DNA ligase (7,17), inhibition of ADPRT might prevent the

rejoining of DNA strand breaks (8) thus increasing the cytotoxicity of radiation.

Normal tissue damage is usually the major limiting factor in any approach to the radiation or medical treatment of malignant tumors. However, NAD^+ pools have been found to be lower in a variety of tumors, fetal tissue and regenerating liver than in normal and adult tissue (48). Therefore it is reasonable to believe that the low NAD^+ pool together with inhibition of ADPRT in connection to DNA damage (i.e. induced by radiation or cytotoxic agents) should result in accumulation of DNA strand breaks which in turn make the combined treatment more toxic for tumor cells than for normal tissue with its higher level of NAD^+ available. Even if the data presented here do not show a specific radiosensitizing effect on tumor cells, the results indicate a possible use of ADPRT inhibitors together with γ -radiation (or cytotoxic agents) in the treatment of malignant tumors.

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TABLE CAPTIONS

- Table 1. The effect of nicotinamide (0.2 g/kg) and γ -radiation at various doses on the growth pattern of C3H mouse mammary adenocarcinoma transplanted to the right leg of the animals.
- Table 2. Nicotinamide-induced tumor volume ratios (C3H tumor volumes on day 30 after treatment).

Days after treatment	Number of animals alive	Average tumor volume $\bar{V} \pm \text{SEM (mm}^3\text{)}$	Significance level of differences in tumor volumes	Animals alive with tumor remission	Animals alive with tumor relapse
A. Untreated controls ⁺					
8	7	1365 $\pm$ 392		0	-
14	4	2247 $\pm$ 469		0	-
28	3	9598 $\pm$ 1259		0	-
B. 5 Gy ⁺					
7	10	844 $\pm$ 217	A vs B NS	0	-
14	9	1266 $\pm$ 343	p < 0.2	0	-
28	8	3162 $\pm$ 880	p < 0.01	0	-
C. Nicotinamide plus 5 Gy ⁺					
8	10	670 $\pm$ 121	B vs C NS	0	-
14	9	1025 $\pm$ 222	NS	0	-
28	6	4198 $\pm$ 552	NS	0	-
D. 10 Gy ⁺					
7	10	385 $\pm$ 108	A vs D p < 0.02	0	-
14	10	1515 $\pm$ 388	NS	1	-
28	6	4592 $\pm$ 1092	p < 0.05	1	-

Table 1, continued

E. Nicotinamide plus 10 Gy ⁺			
7	10	348 ± 55	D vs E NS
14	9	859 ± 200	p < 0.2
28	5	2534 ± 567	p < 0.2
F. 20 Gy (11 animals at the outset)			
7	10	235 ± 45	A vs F p < 0.01
14	10	435 ± 85	p < 0.001
28	10	1312 ± 176	p < 0.001
G. Nicotinamide plus 20 Gy ⁺			
7	10	265 ± 104	F vs G NS
14	10	276 ± 70	p < 0.2
28	10	744 ± 180	p < 0.05
H. 30 Gy ⁺			
8	10	131 ± 31	A vs H p < 0.01
15	10	289 ± 101	p < 0.001
29	10	637 ± 194	p < 0.001
I. Nicotinamide plus 30 Gy ⁺			
8	10	72 ± 32	H vs I [*] NS
15	9	40 ± 24	p < 0.05
29	9	132 ± 62	p < 0.05

Table 1, continued

J. 55 Gy ⁺		A vs J	
7	10	147 ± 35	2
14	10	0	10
28	9	0	9
			-

* The significance level of differences in H vs I on tumor remission is $p < 0.20$ as determined by Mantel Haenzel's test (37). There is also no significant differences in animal survival or number of tumor relapses after the different treatments.

⁺ 10 animals at the outset.

Table 2

γ -ray dose (Gy)	Tumor volume ratios on day 30 ¹
0	0.95 ± 0.30 (NS)
5	0.77 ± 0.28 (NS)
10	1.76 ± 0.54 (NS)
20	1.83 ± 0.49 ($p < 0.05$)
30	4.36 ± 2.30 ($p < 0.05$)

¹
Tumor volume ratios = $\frac{\text{tumor volume on day 30} - \text{NAM}}{\text{tumor volume on day 30} + \text{NAM}}$

CHART LEGENDS

- Chart 1. ^{14}C -activity in various organs versus time after injection of labelled nicotinamide. The arrow indicates the total specific activity given to the animals.
- Chart 2. Average tumor volume versus time after transplantation for the various treatments indicated. The error bars show standard error of the mean. The figures close to the data points indicate the number of animals living. Open symbols give the tumor volumes when no nicotinamide was administered. Filled symbols show tumor volumes in animals given nicotinamide (0.2 g/kg) 30 min before the irradiation.
- Chart 3. Average tumor volumes at day 30 versus dose of γ -radiation. Symbols as in Chart 2. Significance levels of the differences in tumor volumes with and without nicotinamide administered prior to radiation are indicated.
- Chart 4. Number of mitoses per high power field (X500) versus time after treatment with 10 Gy γ -radiation. Open and filled circles give data without nicotinamide administered before the irradiation. Error bars show standard deviation of the data. Significance levels of the differences between the two groups are indicated.
- Chart 5. Tail lengths versus time after treatment for the various doses given. Open circles - no nicotinamide given prior to radiation. Filled circles - animals given nicotinamide (0.2 g/kg) prior to radiation. The curves drawn show the average growth pattern of control animals not given any nicotinamide.

Chart 6. Per cent tail stunting versus dose of γ -radiation for animals with and without pretreatment with nicotinamide. The data points are average values with the error bars indicating standard deviations.

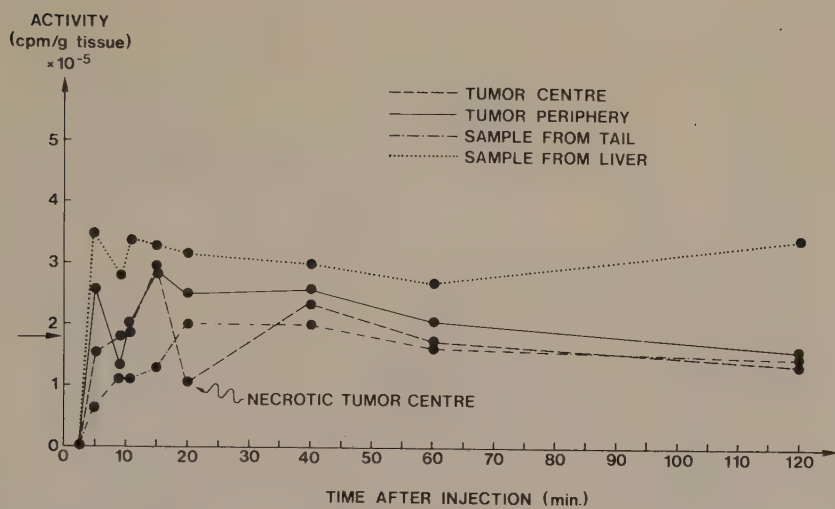


Chart 1

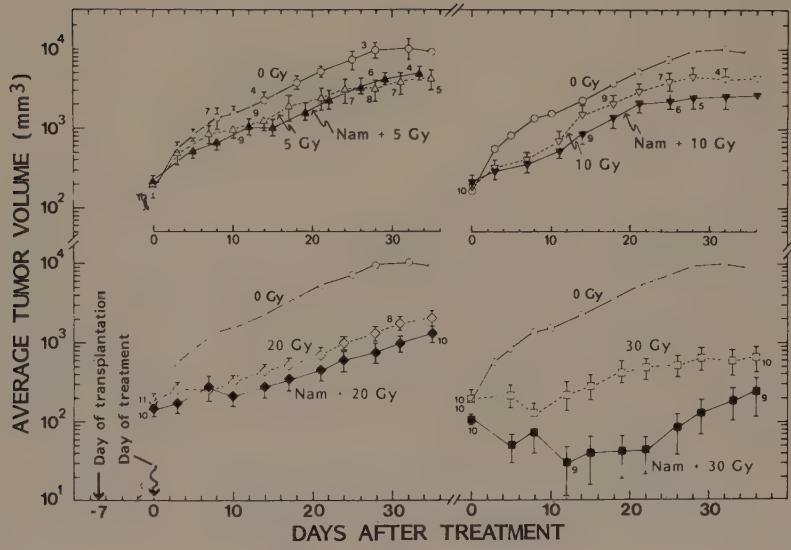


Chart 2

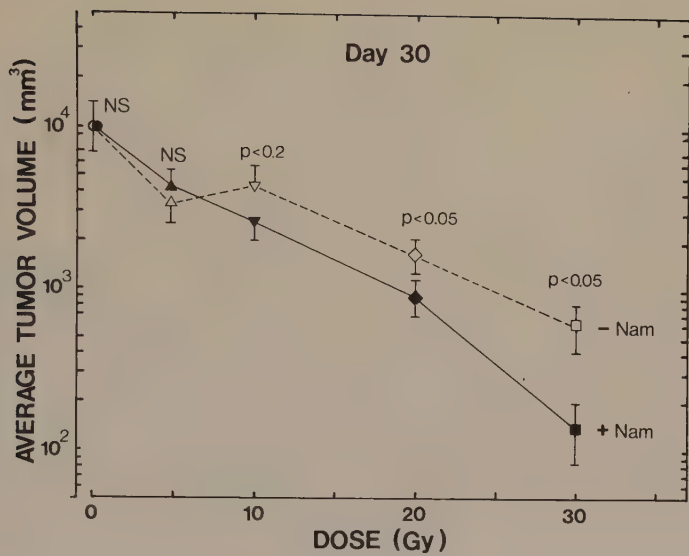


Chart 3

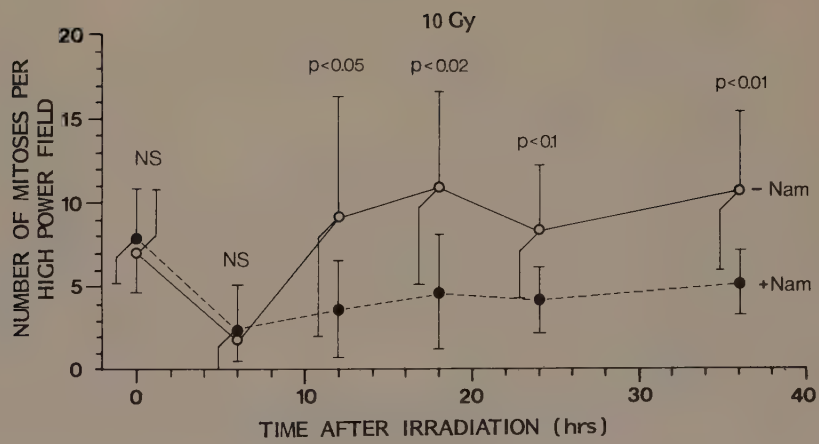


Chart 4

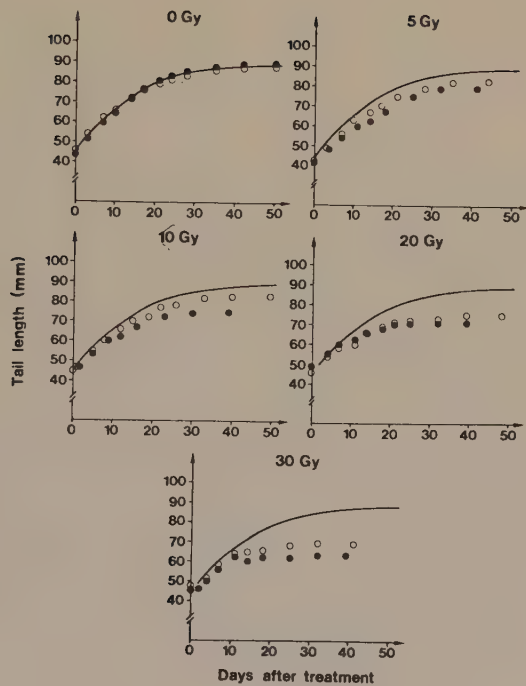


Chart 5

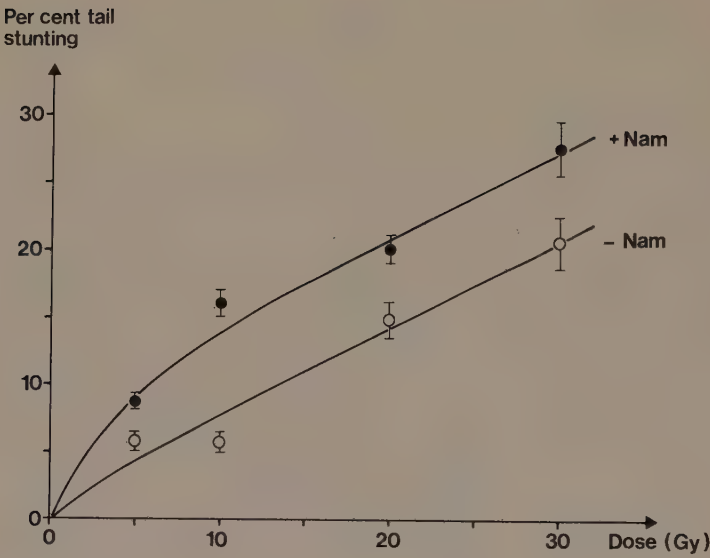


Chart 6

